Lung function responses and mucus secretion in a model of chronic asthma

A thesis submitted to Cardiff University in accordance with the requirements for the degree of

DOCTOR OF PHILOSOPHY

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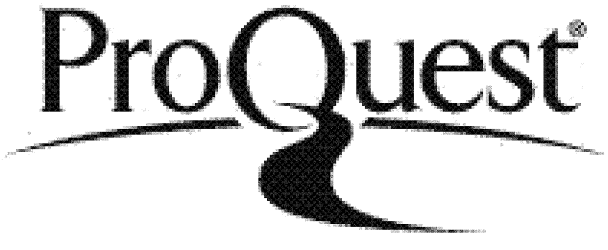

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SUMMARY

Chronic mucus hypersecretion is a pathological feature of several respiratory diseases such as asthma. Although excessive airway mucus production may lead to mucus plugging and contribute to airway obstruction, few studies have investigated an association between mucus hypersecretion and lung function changes in models of asthma.

The acute ovalbumin model of guinea pig asthma induces an early and late asthmatic response, airway hyperreactivity and inflammation, but does not reveal a mucus hypersecretory phenotype. The present studies describe the development of a guinea pig model of chronic asthma, consisting of repeated ovalbumin challenges, which is characterised by an early and late asthmatic response, airway hyperreactivity, inflammation, goblet cell-associated mucin production and mucus hypersecretion. This model was subsequently utilised to study the effect of mucus secretagogues exposures in the airways of guinea pigs with a mucus hypersecretory phenotype.

Lung function responses, epithelial stored mucin levels and mucin secretion were assessed following nebulised exposures of several potential secretagogues including uridine triphosphate, uridine diphosphate, adenosine triphosphate, 5'adenosine monophosphate and histamine. Of these, nebulised exposure to uridine triphosphate (1mM for 15mins) and histamine (10mM for 30mins) (in the presence of mepyramine inhibition (30mg/kg) to prevent against extensive H_1-mediated bronchoconstriction) stimulated both significant goblet cell-associated mucin secretion and a gradual reduction in lung function. It was proposed that in the guinea pig model of chronic allergen challenge, uridine triphosphate and histamine induced goblet cell mucin secretion, resulting in mucus accumulation in the airways and a subsequent reduction in lung function.

In summary, these observations demonstrate that lung function appears to be impaired by mucus secretion in an experimental animal model of chronic allergic asthma. The guinea pig model of chronic asthma may be utilised to investigate the development of a mucus hypersecretory phenotype in asthma or identify potential targets for the development of novel therapies aimed at reducing goblet cell mucus production or secretion.

CONTENTS

ABBREVIATIONS

AB	Alcian blue
ACh	Acetylcholine
ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Antigen presenting cells
ASL	Airway surface liquid
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
CaCC	Calcium activated Cl^- channel
CBF	Ciliary beat frequency
CCSP	Clara cell secretory protein
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CGRP	Calcitonin gene-related peptide
CHD	Chondroitinase
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CysLT	Cysteinyl leukotriene
DAB	3,3'-Diaminobenzidine
DAG	Diacylglycerol
DBA	Dolichos biflorus agglutinin
DNA	Deoxyribonucleic acid
EAR	Early asthmatic response
ECP	Eosinophil cationic protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

ELISA	Enzyme linked immunosorbent assay
ELLA	Enzyme-linked lectin assay
EnaC	Epithelial sodium channel
EPO	Eosinophil peroxidase
ER	Endoplasmic reticulum
GalNAc	N-acetylgalactosamine
GMA	Glycine max agglutinin
HB	Heparin binding
hCLCA1	Ca^{2+} activated Cl^- channel
HNE	Neutrophil elastase
HPA	Helix pomatia agglutinin
HRP	Horse radish peroxidase
HYL	Hyaluronidase
Hz	Hertz
ICOS	Inducible costimulatory receptor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMS	Industrial methylated spirit
IP_3	Inositol phosphate
kg	Kilogram
LAR	Late asthmatic response
Lb	pound
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LT	Leukotriene
LTA	Lipoteichoic acid
M	Molar
mA	milli AMP
MAPK	Mitogen-activated protein kinase
MARCKS	Myristolyated alanine-rich C kinase substrate
MCC	Mucociliary clearance

mg	Milligram
MHC	Major histocompatability complex
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
Mw	Molecular weight
NANC	Non-adrenergic non-cholinergic
NFA	Niflumic acid
NK	Neurokinin
nM	Nanomolar
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
OA	Ovalbumin
OPD	O-phenylenediamine dihydrochloride
P	Purinergic
PAF	Platelet activating protein
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid schiff
PBS	Phosphate buffered saline
PD	Phosphodiesterase
PG	Prostaglandin
PGF	protransforming growth factor
P-gp	P-glycoprotein
PI-3 kinase	Phosphoinositide-3 kinase
PK	Protein kinase
PMA	Phorbol 12-myristate 13-acetate
p.s.i.	Per square inch
PVDF	Polyvinylidene fluoride
RANTES	Regulated upon Activation, Normal T cell-Expressed and Secreted
R_{aw}	Airway resistance

RNA	Ribonucleic acid
s.e.m.	Standard error of mean
SABCpx	Streptavidin-biotin-peroxidase
SDS	Sodium dodecyl sulphate
secs	Seconds
sG_{aw}	Specific airway conductance
SNARE	N-ethylmaleimide sensitive factor receptors
SP	Substance P
TACE	Tumour necrosis factor α converting enzyme
TBS	Tris-buffered saline
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TK	Tyrosine kinase
TNF	Tumour necrosis factor
T_{reg}	Regulatory T cells
TVA	Triticum vulgaris agglutinin
UDP	Uridine diphosphate
UEA-I	Ulex europaeus agglutinin I
UMP	Uridine monophosphate
UTP	Uridine triphosphate
V	Volts
VDAC	Voltage-dependant anion channels
VIP	Vasoactive intestinal protein
VR receptors	Vanilloid receptors
VVL	Vicia Villosa lectin
µl	microlitre

CHAPTER 1

Introduction

1.1 ASTHMA

1.1.1 DEFINITION OF ASTHMA

To define asthma accurately and precisely has been difficult. The term asthma comes from the Greek word 'asthmaino' meaning 'short-breath', and was first used in the years B.C. (Marketos and Ballas 1982). Throughout the 18th and 19th century the definition of asthma has evolved and expanded with the increasing understanding of the mechanisms involved during an asthmatic response. At present, the most commonly quoted definition of asthma comes from the American Thoracic Society (1987):

"Asthma is a clinical syndrome characterised by increased responsiveness of the tracheobronchial tree to a variety of stimuli. The major symptoms of asthma are paroxysms of dyspnoea, wheezing and cough, which may vary from mild and almost undetectable to severe and remitting (status asthmaticus). The primary physiological manifestation of the hyperresponsiveness is variable airway obstruction. This can take the form of spontaneous fluctuations in the severity of obstruction, substantial improvements in the severity of obstruction following bronchodilators or corticosteroids, or increased obstruction caused by drugs or other stimuli"

At present, asthma affects an estimated 5% of the population in the UK (www.asthma.org.uk) and despite significant scientific developments in the treatment of asthma, the increasing prevalence of asthma in industrialised countries has not abated.

1.1.2 PATHOPHYSIOLOGY OF ASTHMA

The presentation of an asthmatic response is a result of a complex cascade of events between cells of the immune system, inflammatory cells, mediators and airway structural cells. In allergic asthma the effector phase of the asthmatic response, which is characterised by the physical symptoms of asthma, is always preceded by the sensitisation phase. This primes the immune system of a genetically predisposed individual to inhaled allergen, so that subsequent exposure will induce an asthmatic response.

1.1.2.1 The sensitisation phase

The process of sensitisation involves the uptake of specific antigens by antigen presenting cells (APC), which frequent the airway epithelium (Palliser *et al* 1998). Once captured, allergens are proteolysed into peptides which complex with major histocompatibility (MHC) class II molecules prior to being transported to the surface of the APC (Banchereau and Steinman 1998). At the cell surface two signals allow the recognition of peptide by naïve CD4+ T lymphocytes (T cells) and subsequent T cell activation: 1.) a signal between the T cell receptor (TCR) of the naïve T cell and the peptide-MHC complex and 2.) a co-stimulatory signal between a CD28 molecule on the T cell and the B7 family of molecules which are expressed on APC (Palliser *et al* 1998). Subsequent to activation, naïve T cells produce IL-2 and develop receptors specific for IL-2 on their cell surface. Activation of cell surface IL-2 receptors induces the proliferation of naïve T cells into either Th1 or Th2 cells, depending on the presence of the autocrine cytokines, IL-12 and IL-4 respectively (Romagnani 1997). In asthmatics, the balance is shifted towards Th2 production.

Activated Th2 cells produce a specific profile of cytokines, which mediate the processes required for allergic sensitisation. IL-4 induces the expression of IgE receptors on mast cells and as well as IL-13, stimulate the production of IgE from B cells. Subsequent fixation of IgE antibodies to mast cell high affinity Fc epsilonRI receptors, specific for IgE, provides an environment whereby re-exposure to the same antigen will induce an allergic (asthmatic) response.

1.1.2.2 Effector phase

The allergic response observed in an atopic individual following re-exposure of a specific allergen consists of both an early asthmatic response (EAR) and a late asthmatic response (LAR). When fixed to mast cells via high affinity Fc epsilonRI receptors (subsequent to sensitisation), the half-life of IgE antibodies can be increased 10fold (Holmes 1999). The mast cell-fixed IgE antibodies facilitate the activation of mast cell by specific allergen following re-exposure of allergen or if any allergen is remaining in the body subsequent to the initial exposure. This results in rapid mast cell degranulation (60-300 secs) (Holmes 1999) and the subsequent release of multiple inflammatory mediators including histamine, cysteinyl leukotrienes, prostaglandins,

trypase, chymase, cytokines and chemokines. Mast cell-derived mediators, particularly histamine, activate local receptors resulting in bronchoconstriction and presentation of the EAR.

Many of the inflammatory mediators released following mast cell degranulation are potent chemotaxins, and promote the recruitment of inflammatory cells, such as macrophages, eosinophils and neutrophils into the lungs, which release additional inflammatory mediators and contribute to the LAR. Eosinophilia is strongly indicated in the late phase asthmatic response (Durham and Kay 1985). Additionally, basophils, platelets, epithelial cells, smooth muscle cells, endothelial cells and fibroblasts are also capable of synthesising and releasing inflammatory mediators (Barnes *et al* 1998). There are a host of mediators involved in the asthmatic response, far beyond the scope of this thesis. However, many of these mediators are summarised in Table 1.1.

1.1.2.2.1 Airway remodelling

Airway remodelling can be defined as the structural changes revealed in the airway following inflammatory responses that can have an effect on the functional activity of the lungs. Airway remodelling can be extensive and irreversible, affecting all components of the epithelial wall, including the epithelium, submucosa, smooth muscle and vascular structures. The pathological changes include thickening of the epithelium and submucosa, goblet cell hyperplasia, submucosal gland hypertrophy, mucus hypersecretion, oedema (via an increase in the permeability of blood vessel membranes), angiogenesis and increased smooth muscle mass (via myofibroblast hyperplasia) (Jeffery 2001). Airway remodelling results in a reduction in the diameter of the airway lumen as a result of airway wall thickening, oedema and mucus accumulation. This may contribute to airway hyperreactivity (AHR) and a loss of lung function.

AMINE MEDIATORS	PROTEASES
Histamine Serotonin (5-HT) Adenosine	Mast cell tryptase Mast cell chymase Matrix metalloproteinases
PEPTIDE MEDIATORS	**LIPID DERIVED MEDIATORS**
Bradykinin Tachykinins Calcitonin gene-related peptide (CGRP) Endothelins Complement	Prostanoids Leukotrienes Platelet activating factor (PAF) Hydroperoxyeicosatetraenoic acid (HPETEs) Lipoxins (LXs)
CHEMOKINES	**SMALL MOLECULES**
CC chemokines CXC chemokines	Reactive oxygen species Nitric oxide (NO)
CYTOKINES	
Lymphokines *e.g. Interleukin (IL)-2, IL-3, IL-4, IL-5, IL-13, IL-15, IL-16, IL-17.* Proinflammatory cytokines *e.g. IL-1, tumour necrosis factor (TNF-α) IL-6, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF)* Inhibitory cytokines *e.g. IL-10, IL-1 receptor antagonist (IL-1a), interferon-γ (IFN- γ), IL-12, IL-18.* Growth factors *e.g. platelet derived growth factor (PDGF), transformaing growth factor-β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF)*	

Table 1.1. Inflammatory mediators of asthma. Summarised from Barnes *et al* (1998).

1.1.2.2.2 Airway Hyperreactivity (AHR)

The asthmatic airway reveals increased responsiveness to a variety of stimuli (American Thoracic Society 1987). Hyperresponsiveness can be specific (to stimuli that elicit an immune response) or non-specific (to stimuli such as histamine, methacholine or cold air). Non-asthmatics do not respond to specific stimuli. However, they do respond to non-specific stimuli, but require greater doses and elicit a smaller response compared to asthmatics.

Histamine was one of the first mediators implicated in the pathophysiology of asthma. In 1911, Dale and Liadlaw demonstrated the induction of anaphylaxis in laboratory animals following histamine exposure (Dale and Liadlaw 1911) and later in 1946, Curry examined the effects of increasing doses of inhaled histamine in subjects with and without asthma (Curry 1946). In the asthmatic airway, airway narrowing in response to histamine occurs at lower concentrations of histamine compared to the non-asthmatic airway (AHR), as can be identified as a shift to the left of a dose-response curve. AHR to inhaled histamine is now an important diagnostic tool clinically and an important indicator of asthmatic status in the laboratory.

The precise mechanisms involved in the development of AHR are unclear. However components of the inflammatory response and airway remodelling are thought to contribute to AHR. As discussed earlier, airway remodelling and inflammation can lead to a reduction in the diameter of the airway lumen, due to airway wall thickening, oedema, mucus hypersecretion and reduced external support of the airway wall (Rogers 2004). As a result of the reduction in airway luminal diameter, an amplified response to a bronchoconstrictor, such as histamine, can be observed in remodelled airways (Lambert *et al* 1993). Additionally, airway obstruction due to mucus accumulation and plugging may contribute to AHR. The pattern of mucus distribution in the airways is usually not homogenous, resulting in restriction of certain parts of the airways and uneven airflow. This may lead to amplified response to inhaled bronchoconstrictors (Rogers 2004). Furthermore, exposure of receptors and nerves in the airway wall, as a result of epithelial shedding following airway inflammation, may result in increased bronchoconstriction following inhaled histamine.

1.2 LUNG STRUCTURE

The structure of the respiratory system and morphology of respiratory cell types has been reviewed extensively (Godwin 2002, Bowen 1998, LeMaistre 2003, Rogers 1994, Mason 2005). The respiratory system consists of a complex structure of tubular passageways, which facilitates both inspiration and expiration of air and allows intermediate gas exchange. The trachea, bronchi, bronchioles and terminal bronchioles form the conducting portion of the respiratory system which permits transport of gases, while the respiratory bronchioles and the alveoli form the respiratory portion, which allow gas exchange between air and the circulatory system.

The trachea is a semi-rigid tube, which branches into 2 bronchi. Each bronchi divides further into 3 right lobar bronchi and 2 left lobar bronchi, which supply the 3 right pulmonary lobes and 2 left pulmonary lobes respectively. The lobar bronchi branch further into segmental bronchi, bronchioles, terminal bronchioles and finally respiratory bronchioles. As the branching of the lower respiratory system becomes more extensive, gradual changes are seen in the structure of the airway wall.

1.2.1 AIRWAY WALL

The epithelium forms the interior lining of the respiratory system. It consists of a layer of morphologically distinct, closely packed epithelial cells and is separated from the connective tissue by a thin basement membrane. The basement membrane itself consists of one layer of protein filaments and one layer of reticular fibres, named the basal lamina and the reticular lamina respectively and lies adjacent to the poorly defined lamina propria, which is a combination of blood vessels, nerve bundles and free cells. Vital support is provided firstly by the connective tissue of the submucosal layer and further, by rings or plates of cartilage and smooth muscle, which encircle the submucosa. Fig. 1.1 represents the layers of cells and tissue in the airway wall.

1.2.2 EPITHELIUM

The airway epithelium provides the initial site of contact between the external environment and the lungs. It is essential therefore, that the structure of the airway epithelium allows optimum gas exchange without compromising its protective role. As

the branching of the respiratory epithelium becomes more extensive, changes are observed in the structure of the airway epithelium. In the trachea and bronchioles, the epithelium takes the form of typical respiratory epithelium (TRE), which consists of pseudostratified ciliated columnar epithelium dispersed with goblet cells (Jeffery and Li 1997). However, the airway epithelium evolves into simple columnar epithelium and simple cuboidal epithelium in the distal and the respiratory bronchioles respectively.

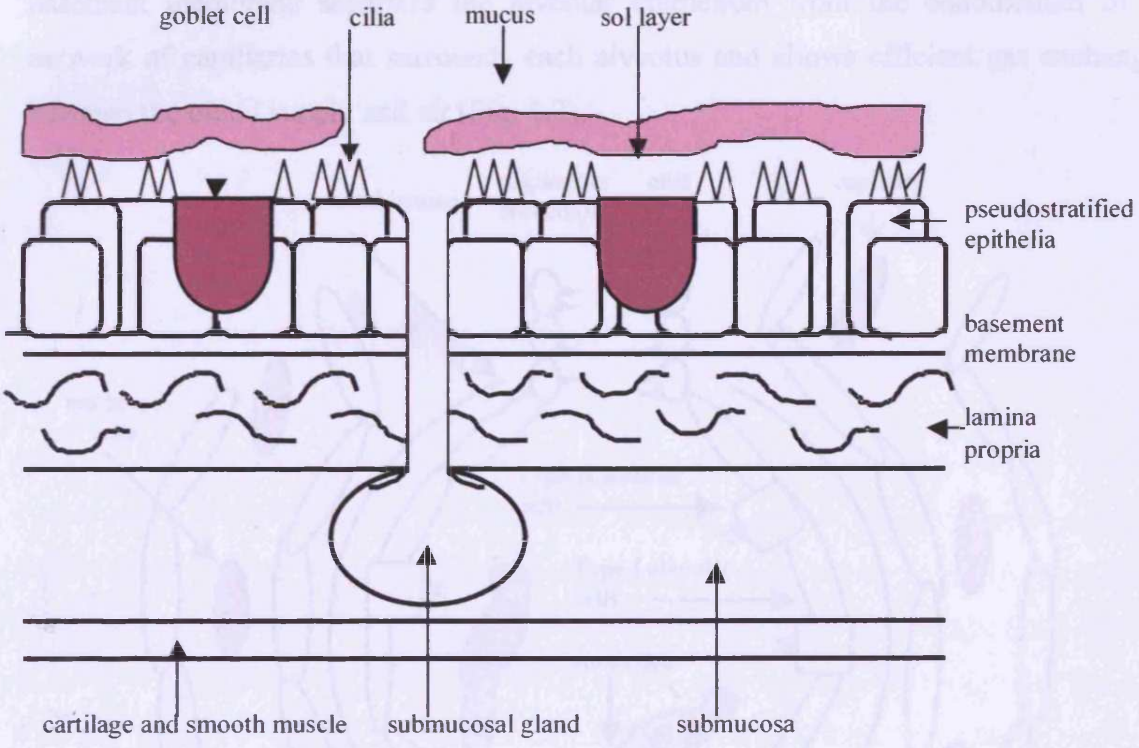


Figure 1.1 Schematic diagram of the human bronchiole airway wall

1.2.3 CARTILAGE AND SMOOTH MUSCLE

Cartilage and smooth muscle reinforce the structure of the respiratory system, and allow the airways to expand and recoil during inspiration and expiration respectively. The content of cartilage and smooth muscle in the airways is inversely proportional to one another. In the trachea and bronchi, cartilage forms C-shaped rings composed of hyaline, which are separated from one another and supported by small quantities of smooth muscle (LeMaistre 2003). However, as branching of the respiratory system becomes more extensive, irregular plates of cartilage replace the C-shaped cartilage

rings until eventually, in the smallest bronchiole, support is provided solely by smooth muscle and connective tissue (LeMaistre 2003).

1.2.4 ALVEOLI

Alveoli are sac-like structures, which bud out of the epithelial wall in the respiratory bronchioles and form the main areas of gas exchange in the respiratory tract. A thin basement membrane separates the alveolar epithelium from the endothelium of a network of capillaries that surrounds each alveolus and allows efficient gas exchange between the blood supply and air (Fig. 1.2).

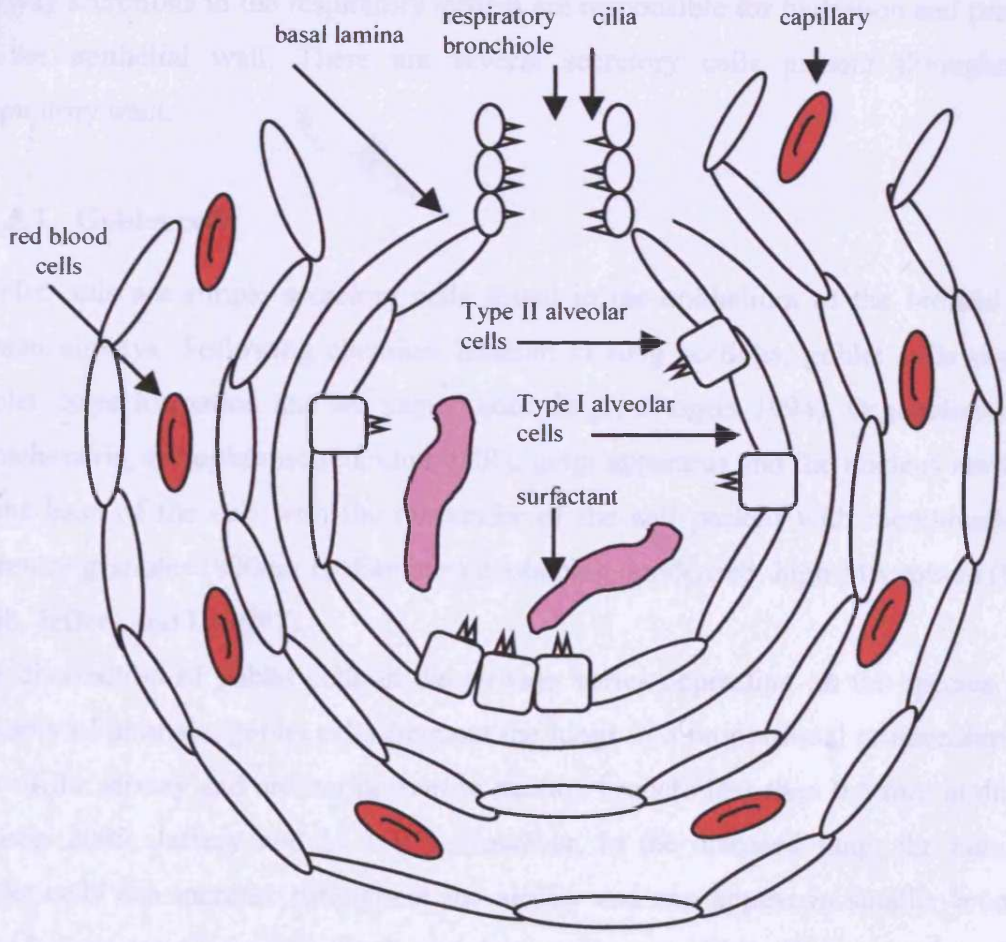


Figure 1.2. Schematic diagram of an alveolus

The epithelium of the alveoli sacs is simple and consists of 2 cell types: Type I alveoli cells and Type II alveoli cells. Squamous shaped Type I alveoli cells make up 40% of the alveoli cells, but account for 90% of the total alveolar surface area. This is the main

area of gas exchange in the alveoli (Godwin 2002, LeMaistre 2003). The remaining 60% of alveoli epithelial cells are rounded Type II alveoli cells (Godwin 2002), which have the capacity to transform into alveoli Type I cells, following damage to the lung (LeMaistre 2003). These cells also possess microvilli and granules containing the surfactant lecithin, which is released onto the surface of the alveoli to protect the epithelium and reduce surface tension (Godwin 2002). There are, however, no goblet cells, glands or Clara cells present in the alveoli (LeMaistre 2003).

1.2.5 RESPIRATORY SECRETORY CELL PHENOTYPES

Airway secretions in the respiratory system are responsible for hydration and protection of the epithelial wall. There are several secretory cells present throughout the respiratory tract.

1.2.5.1 Goblet cells

Goblet cells are simple secretory cells found in the epithelium of the bronchi of the human airways. Following chemical fixation of lung sections, goblet cells possess a goblet shape formation and are named accordingly (Rogers 1994). Organelles such as mitochondria, endoplasmic reticulum (ER), golgi apparatus and the nucleus are located at the base of the cell with the remainder of the cell packed with membrane-bound secretory granules (800nm in diameter) containing condensed, high Mw mucin (Bowen 1998, Jeffery and Li 1997).

The distribution of goblet cells in the airways varies depending on the species. In the majority of animals, goblet cells frequent the lungs in a proportional relationship to the size of the airway and are not present in healthy bronchi less than 0.5 mm in diameter (Mason 2005, Jeffery and Li 1997). However, in the diseased lung, the number of goblet cells can increase throughout the airway and can appear in smaller bronchi to provide a source of mucus in the lower airways (Rogers 1994). The mechanisms behind this increase in goblet cell number are unclear, but it is considered that goblet cells are formed from the differentiation, rather than the proliferation, of epithelial cells, possibly Clara cells (metaplasia) (Takeyama *et al* 1999).

1.2.5.2 Clara cells

No secretory glands and very few goblet cells are present in the distal bronchioles (LeMaistre 2003). However, small granular cells called Clara cells are located in the epithelia of distal bronchioles (Jeffery and Li 1997). Although the exact function of these cells is unclear, they produce a mucus-like watery secretion containing a 16kD homodimeric protein called Clara cell secretory protein (CCSP) (Mason 2005). The secretion is thought to combine with surfactant in the alveoli to reduce surface tension, and to combine with mucus in the bronchi to aid mucociliary clearance (Godwin 2002). CCSP expression is decreased in asthmatic airways (Mason 2005) and the number of Clara cells decreases following goblet cell metaplasia in rats (Takeyama *et al* 1999). Additionally, a small proportion of goblet cells secrete CCSP, prompting the suggestion that Clara cells may function as precursor cells for goblet cells (Boers *et al* 1999).

1.2.5.3 Submucosal cells

The submucosa of the airway wall is populated with additional secretory cells: the cells of the submucosal glands (Fig. 1.1). The submucosal glands frequent the airways in the trachea and bronchial airways with a diameter greater than 2mm (Ballard & Inglis 2004). In healthy airways, the submucosal glands outnumber the goblet cells by 20fold (Lamb and Reid 1972) and supply an estimated 95% of airway mucus (Reid 1960). The submucosal glands are more complicated in structure compared to goblet cells, and were described in detail by Meyrick and Reid (1970). The primary duct of the submucosal gland extends from the submucosa and is continuous with the epithelium of the airway surface, allowing efficient passage of gland secretions into the airway lumen. The main body of the submucosal gland consists of a collecting duct, which empties secretions into the primary duct and controls ionic and water concentrations in the gland secretions (Wine and Joo 2004). Projecting from the collecting duct are two lateral ducts, which serve to collect secretions from multiple projecting secretory tubules (Ballard and Inglis 2004). Two types of secretory cells are located in acini surrounding the secretory tubules of the submucosal gland: the serous and mucous cells, which contain fluid-filled granules and mucus-filled granules respectively. Secretory tubules can be named serous or mucous tubules, according to the relative predominance of the respective cell type. Mucous cells are present in their proximal regions of the secretory

tubules, whilst serous cells are present in their distal regions (Wine and Joo 2004). This appears to facilitate the effective emptying of the submucosal glands. Fluid secretions from serous cells located on the outermost portions of the secretory tubules are thought to flush the mucous tubules and aid the removal of mucus from the submucosal glands into the airways. In health, submucosal gland secretion is a mixture of water, mucus, electrolytes and a mixture of antimicrobials, anti-inflammatories and antioxidants, and submucosal gland cells are present at an approximate ratio of 3 serous cells to every 2 mucous cell (Wine 2007, Meyrick and Reid 1970). However in lung disease, submucosal glands can increase in size (hypertrophy) and increase in the relative proportion of mucous cells to serous cells.

The airways are innervated with autonomic nerves, which locate close to the submucosal glands, and appear to regulate mucus and fluid secretions. Submucosal gland mucus secretion appears to be under parasympathetic control in all animals and under both parasympathetic and sympathetic control in smaller animals (Rogers 2000). Additionally, submucosal glands are associated with myoepithelial cells, which are found beneath the mucous and serous tubules (Meyrick and Reid 1970). The cells are contractile and function to squeeze mucus and facilitate emptying of the contents of the secretory tubules (Shimura *et al* 1986).

1.3 MUCUS

1.3.1 PROPERTIES OF MUCUS

The airways are repeatedly exposed to dangerous or irritant particles and bacteria in the air we breathe. The airway surface liquid (ASL) is a vital defence mechanism and is essential to maintain healthy functioning of the lung. The human ASL consists of an overlying mucus layer (~55μm in thickness) and a periciliary sol layer (7μm in thickness) (Jayaraman *et al* 2001, Krouse 2001) (Fig. 1.1). In health, the mucus layer functions as a network to trap particles and bacteria in the airways, whilst the sol layer maintains an optimum distance between the mucus layer and the epithelium, in order to protect the epithelium and provide a low-viscosity solution in which cilia can beat. Airway mucus can be cleared from the airways, facilitated either by beating cilia (which waft the mucus towards the mouth to be swallowed) or by cough (which dislodges mucus from the airways) (Rogers 1994).

Mucus is secreted into the lungs by airway secretory cells, of which there are 2 main types: the mucous cells of the submucosal glands (only present in large airways/ cartilaginous airways) and the surface epithelial goblet cells (present in the large airways and small/non-cartilaginous airways). Mucus is a complex mixture, consisting mainly of water (95%) with the remaining 5% made up of electrolytes, macromolecules (mucin glycoproteins, N-glycoproteins and proteoglycans), lipids, DNA, antibodies, serum proteins, lactoferrin, defensins and antiproteases.

The major macromolecular component of mucus is the high Mw glycoprotein, mucin (Jackson 2001). Mucins are threadlike molecules and are polydisperse in size (from 3 to 32 million Dalton) and length (0.5μm-10μm) (Henke *et al* 2004, Thornton *et al* 1990). Specific MUC genes, of which more than twenty human mucin genes have been cloned to date and named MUC1-20 accordingly, encode the peptide backbones of mucins. The MUC genes can be divided into secreted and membrane-tethered mucins. Secreted mucins can further be divided into gel-forming and non gel-forming mucins. Only five of these MUC genes encode for the gel-forming mucins, which form the sticky mucus present in the airway lumen: MUC2, MUC5AC, MUC5B, MUC6 and MUC19 (Lidell and Hansson 2006). Of these, MUC5AC and MUC5B dominate in human sputum and together make up the majority of the gel-forming mucins in the airways (Kirkham *et al*

2002). MUC5AC is the major gene that is expressed in goblet cells (Fahy 2002) and produces the smaller gene product (Hovenberg *et al* 1996). MUC5B is the major gene that is expressed in submucosal glands, but is also present in goblet cells (Groneberg *et al* 2002, Rogers 2003). Membrane-tethered mucins are attached to the membranes of epithelial cells via a hydrophobic membrane-spanning domain. The specific function of these mucins is unclear. However, because of their specific location in the airway, it is probable that as well as functioning as a simple protective epithelial barrier, they may also function as cell-surface receptors, regulating several cellular functions such as innate defence or epithelial repair (Voynow 2002, Wreschner *et al* 1994). Furthermore, mucus consists of protective compounds including anti-microbials, anti-inflammatories eg lysozyme, lactoferrin, siderocalin, pore-forming defensins and lactoperoxidase and immune cells (natural killer cells, cytotoxic T cells, macrophages and neutrophils) (Wine 2007).

1.3.2 STRUCTURE AND FORMATION OF GEL-FORMING MUCINS

The translation of MUC genes into mucin peptides has been reviewed in several papers (Rose *et al* 2001, Van den Steen 1998). Translation of the MUC genes results in the formation of a protein backbone, composed of serine- and threonine-rich tandem repeat domains of amino acid sequences and cysteine-rich domains at the amino and carboxyl termini (Rose 1992). The terminal cysteine-rich domains provide essential sites for disulphide linkages between mucin peptide subunits, whereas the serine and threonine-rich tandem repeat domains provide sites for o-glycosylation (Rose and Voynow 2006) (Fig. 1.3).

O-glycosylation is a post-translational event, resulting in the attachment of numerous oligosaccharides via o-glycosidic bonds between a N-acetylgalactosamine (GalNAc) group and the hydroxyl moieties of serine and threonine of the mucin backbone (Rose 1992). O-glycosylation begins with the addition of a single GalNAc to any serine or threonine on the mucin backbone by a family of GalNAc transferases. Following addition of the initial GalNAc, glycosylation by a series of glycosyltransferases results in the formation of core structures, which are then modified by sialylation, fucosylation, sulphation, methylation and acetylation into complex oligosaccharides (Rose 1992, Van den Steen 1998). Each oligosaccharide contains a combination of five sugar types: N-

acetylgalactosamine, N-acetylglucosamine, galactose, sialic acid and fucose. They can be linear or branched and vary in size, but are always sulphated and terminate in sialic acid (Rogers 1994).

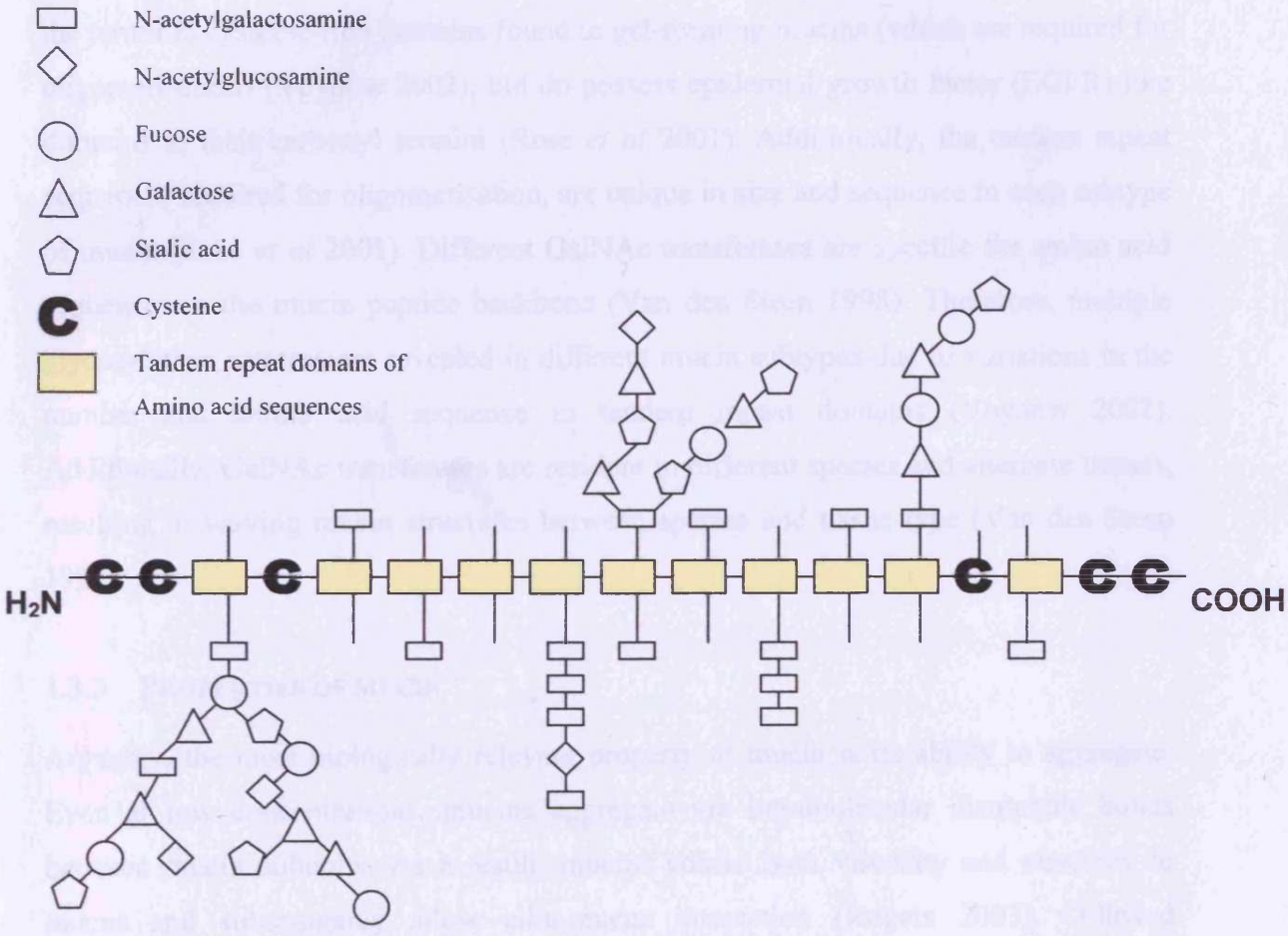


Figure 1.3. Theoretical structure of a mucin unit.
Mucins are high molecular weight glycoproteins consisting of a peptide backbone and o-glycosidic-linked oligosaccharides. The peptide backbone is composed of tandem repeat domains of amino acid sequences and cysteine rich domains at the amino and carboxyl termini, which provide sites for o-linked glycosylation and disulphide bonds for oligomerisation respectively. Oligosaccharide units consist of different combinations of the sugars, N-acetyl galactosamine, N-acetylglucosamine, sialic acid, fucose and galactose. Oligosaccharides can be linear, branched and can vary in size. Oligosaccharides are linked to the peptide backbone by o-glycosidic linkage between N-acetylgalactosamine and serine or threonine of the peptide repeat domains (Voynow *et al* 2002, Rose *et al* 1992).

Different MUC genes encode for different mucin subtypes and the structure of an individual mucin differs depending on the MUC gene it is encoded by. For example, the gel-forming mucins do not possess the hydrophobic transmembrane domain found in membrane-tethered mucins. Furthermore, the membrane-tethered mucins do not possess the terminal cysteine-rich domains found in gel-forming mucins (which are required for oligomerisation) (Voynow 2002), but do possess epidermal growth factor (EGFR)-like domains at their carboxyl termini (Rose *et al* 2001). Additionally, the tandem repeat sequences required for oligomerisation, are unique in size and sequence in each subtype of mucin (Rose *et al* 2001). Different GalNAc transferases are specific for amino acid sequences in the mucin peptide backbone (Van den Steen 1998). Therefore, multiple glycosylation patterns are revealed in different mucin subtypes due to variations in the number and amino acid sequence in tandem repeat domains (Voynow 2002). Additionally, GalNAc transferases are resident in different species and alternate tissues, resulting in varying mucin structures between species and tissue type (Van den Steen 1998).

1.3.3 PROPERTIES OF MUCIN

Arguably, the most biologically relevant property of mucin is its ability to aggregate. Even at low concentrations, mucins aggregate via intramolecular disulphide bonds between mucin subunits. As a result, mucins confer both viscosity and elasticity to mucus and subsequently allow cilia-mucus interaction (Rogers 2003). O-linked glycosylation is also important for protein stability and may confer heat and protease resistance (Van den Steen 1998, Kramerov *et al* 1996).

In addition to giving mucus its characteristic viscoelasticity, mucin oligosaccharides can adhere to bacteria (Vishwanath and Ramphal 1984). Bacteria, such as *Pseudomonas aeuruginosa* are associated with airway infections in cystic fibrosis (CF) lungs and may contribute to further inflammation (Jansen *et al* 1999).

1.3.4 GOBLET CELL HYPERPLASIA AND MUC GENE UPREGULATION

In disease, goblet cells have the capacity to increase dramatically in number, a process known as hyperplasia. Additionally, MUC gene expression can be upregulated and

goblet cells can increase substantially in size, resulting in the increased production of goblet cell-stored mucin and increased mucin secretion.

1.3.4.1 Goblet cell hyperplasia

Understanding the exact pathways involved in goblet cell hyperplasia, particularly in disease, is vital to identify potential targets for the development of novel therapies aimed at reducing goblet cell mucus secretion. However, the precise mechanisms involved in goblet cell hyperplasia are unclear. There is convincing evidence to suggest that increases in epithelial goblet cell numbers are a result of differentiation of non-granular progenitor cells (metaplasia) and not a result of cell proliferation (Rogers 2003). For example, studies by Takeyama *et al* (1999) revealed that induction of goblet cell hyperplasia by TNF-α in rat airways, was not associated with alteration in total epithelial cell numbers, indicating differentiation of existing cells, rather than proliferation of one cell into multiple goblet cells. The proposed progenitor cell was the non-ciliated epithelial cell, the Clara cell, due to observations that in diseased airways, the number of Clara cells is inversely proportional to the number of goblet cells. Additionally, the Clara cell secretes Clara cell secretory protein, (CCSP) into the airways, which can also be secreted by some goblet cells (Boers *et al* 1999).

1.3.4.2 Goblet cell metaplasia, EGFR and IL-13

The epidermal growth factor receptor (EGFR) and signalling via IL-13 both appear to be of significant importance in goblet cell metaplasia. The EGFR is a 170kDa membrane glycoprotein and is thought to be involved in cell proliferation, differentiation, motility and survival. EGFR expression is sparse in the airways of normal subjects, but can be significantly increased in asthmatic airways and following various irritant stimuli (Takeyama *et al* 2001).

Takeyama *et al* (1999) studied the effect of TNF-α and EGFR tyrosine kinase (TK) inhibitors on both a mucin-producing cell line (NCI-H292) and *in vivo* in rats. They have suggested that in rat airways, expression of EGFR on Clara cells can be activated by proinflammatory mediators such as TNF-α. Activation of EGFR stimulates EGFR tyrosine phosphorylation and mitogen-activated protein kinase (MAPK) signalling,

ultimately resulting in the differentiation of the Clara cell into goblet cells and the formation of mucin granules.

However, IL-13 also appears to be a significant activator of goblet cell metaplasia (Atherton *et al* 2003) and recently, studies by Tyner *at al* (2006) have demonstrated that both EGFR and IL-13 signalling are necessary for goblet cell metaplasia and mucus production. They suggest that activation of the EGFR on airway epithelial cells inhibits apoptosis, thus allowing IL-13-stimulated differentiation of ciliated cells into goblet cells.

1.3.4.3 Goblet cell metaplasia and additional stimuli

Many Th2 cell-derived cytokines induce goblet cell metaplasia, including IL-4 (Dabbagh *et al* 1999), IL-5 (Justice *et al* 2002), and IL-13 (Atherton *et al* 2003), and PGE_2 (Borchers *et al* 1999). Additionally, platelet activating factor (PAF) has also been shown to stimulate goblet cell metaplasia (Komori *et al* 2001). However, further research is required to understand the mechanisms of Th2 inflammatory mediator-induced goblet cell metaplasia and the interaction of the Th2 cytokines and the EGFR system.

1.3.4.4 MUC gene upregulation

Multiple stimuli have been shown to upregulate MUC gene expression by either increasing MUC gene transcription and/or increasing MUC gene mRNA stability. Inflammatory mediators important in asthma appear to play a vital role in upregulation of MUC gene expression including IL-9 (Longphre *et al* 1999), IL-8, (Bautista *et al* 2001), neutrophil elastase (Fischer and Voynow 2002), IL-1β and TNF-α, as well as lipopolysaccharide (LPS) (Koo *et al* 2002) and acrolein (Borchers *et al* 1999).

The EGFR also appears to be of significant importance in the upregulation of MUC gene expression and mucus production. The EGFR can be activated by a variety of ligands, such as EGF, transforming growth factor (TGF-α), heparin binding (HB) EGF, amphiregulin and epiregulin. These ligands are synthesised as transmembrane proligands, which can be cleaved by enzymes to release the mature ligand (Burgel and Nadel 2004).

The enzymes, TACE (tumour necrosis factor α converting enzyme) and ADAM-10 are both members of the ADAM (A disintegrin and metalloproteinase) family of zinc-dependent transmembrane metalloproteinases, and can cleave EGFR proligands (pro-TGF-α and pro-HB-EGF respectively) into active ligands (Mason 2005). Activation of either enzyme can result in EGFR activation and mucus production. ADAM10 can be activated by lipoteichoic acid (LTA), a component of the cell walls of bacteria, resulting in the cleavage of proHB EGF (Lemjabbar and Basbaum 2002). Activation of TACE and subsequent cleavage of pro-TGF-α, can be stimulated by phorbol 12-myristate 13-acetate (PMA), LPS and P aeruginosa (Shao *et al* 2003).

Cigarette smoke has been shown to induce cleavage of EGFR proligands on epithelial cells, resulting in the release of the mature ligand and subsequent activation of the EGFR (Shao *et al* 2004). Mucin production is also increased in association with airway recruitment of inflammatory cells. Inflammatory cells, such as neutrophils, macrophages and eosinophils express EGFR ligands on their cell surfaces and can release inflammatory mediators, such as free radicals, TNF-α, neutrophil elastase and TGF-α (eosinophils), which are capable of activating EGFR directly and indirectly (Kim and Nadel 2004)

Activation of the purinergic receptors may also stimulate increases in mucus production. Uridine triphosphate (UTP) can stimulate increases in MUC gene expression via activation of the $P2Y_2$ receptor, or possibly $P2Y_4$ or $P2Y_6$ receptor activation (Chen *et al* 2001).

1.3.5 GOBLET CELL-ASSOCIATED MUCIN SECRETION

Mucus is stored in condensed form in granules of goblet cells. Its polyanionic charges, due to its sialic acid moieties on the oligosaccharide side chains of the mucin molecule, are neutralised by calcium ions when in stored form (Rogers 1994). Secretion of mucus from goblet cells is via exocytosis. This involves movement of mucin granules to the internal surface of the apical membrane, fusion of membranes and opening of the granule (Rogers 2003). During exocytosis, a calcium/potassium exchange causes water to enter the granule. Mucin can expand up to 500 fold and is released from the goblet cell. Exocytosis is a fast process and results in the immediate discharge of large quantities of mucus (Lethem *et al* 1993). Although there is a consistent low level

secretion of mucus from the goblet cells, stimuli can cause a drastic increased level of mucus secretion. It was suggested by Basbaum *et al* (1999) that the respiratory epithelium may have the capacity to upregulate mucin secretion in an emergency in addition to secreting a baseline volume of mucin.

Several mediators and compounds stimulate secretion of mucins, possibly via multiple intracellular signalling pathways and with different kinetics (Rose *et al* 2001). Exocytosis of mucin granules from goblet cells appears to be mediated by myristolyated alanine-rich C kinase substrate (MARCKs). The activation of goblet cell surface receptors by secretagogues results in the activation of protein kinases C and G (PKC and PKG) which cooperate to stimulate exocytosis. Phosphorylation of MARCKS by PKC induces its translocation into the cytoplasm of the goblet cell. PP2A (which is activated by PKG via a nitric oxide (NO)-cGMP pathway) subsequently dephosphorylates MARCKS, and this permits attachment of MARCKs to both the mucus granule and to the actin and myosin machinery in the cell. This facilitates movement of the granule to the cell membrane (Mason 2005). Experiments using inhibitor peptides of MARCKs have shown that inhibiting MARCKs can inhibit mucin exocytosis from mucus granules (Singer *et al* 2004). Docking and membrane fusion of mucin granules with the goblet cell membrane is likely to involve N-ethylmaleimide sensitive factor receptors (SNARE), Sec1/Munc18 homologs and Rab protein (Rogers 2003).

1.3.6 GOBLET CELL MUCUS SECRETION/SECRETAGOGUES

A myriad of substances can stimulate the release of mucus from goblet cells and the mucous cells of submucosal glands. However, multiple receptor subtypes appear to be involved and it is unclear what exact mechanisms are responsible for inducing mucus secretion. Below, I will discuss some of these secretagogues.

1.3.6.1 Parasympathetic nervous system

The parasympathetic nervous system is the dominant pathway controlling mucus secretion in human airways and all mammals. Parasympathetic nerves innervate the airways at the submucosal glands and vagal nerve activation can stimulate submucosal gland mucus secretion via muscarinic receptors (Seale 2003). Muscarinic receptors are

also densely found in submucosal glands and moderately found in nerves and smooth muscle (van Koppen *et al* 1988, Mak and Barnes 1990). In several species including humans and ferret, activation of the M3 receptors, which localise in human submucosal glands, stimulates submucosal gland-associated mucin secretion (Ishihara *et al* 1992, Ramnarine *et al* 1996). Goblet cell mucin secretion is also under parasympathetic control. The neurotransmitter ACh can stimulate goblet cell mucus secretion (Farley and Dwyer 1991) and parasympathetic vagal-mediated goblet cell mucus secretion has been demonstrated in guinea pigs (Tokuyama *et al* 1990)

1.3.6.2 Sympathetic nervous system

Adrenergic nerve fibres are also located close to submucosal glands in a number of species (Rogers 2000). β-adrenergic receptors are widely distributed in the airways and present on the surface of epithelial cells and secretory cells (Carstairs *et al* 1985). In the guinea pig, mucus secretion was partly inhibited by propranolol, suggesting a possible role for adrenergic (β) stimulation of mucus (Tokuyama *et al* 1990). Additionally, in the cat, α-adrenergic agonists (and to a lesser degree, β-adrenergic agonists) appear to stimulate mucus secretion (Phipps *et al* 1980). However, although adrenergic nerves may stimulate mucus secretion in mammals, they do not appear to stimulate mucus secretion in human airways (Baker *et al* 1985).

1.3.6.3 Non-adrenergic, non-cholinergic (NANC) neurotransmitters:

Several neurotransmitters of the non-adrenergic non-cholinergic (NANC) nervous pathway reveal effects on airway mucus secretion in different species. Substance P (SP) can stimulate mucus secretion in the human bronchi (Rogers *et al* 1989). Studies using ferret trachea have shown that SP, Neurokinin A (Ramnarine *et al* 1994) and VIP (Kim *et al* 2006) all stimulate mucus secretion in ferret trachea, whereas NO appears to either have no effect (Kim *et al* 2006) or may inhibit mucus secretion (Ramnarine *et al* 1996).

1.3.6.4 Purinergic receptor agonists

The $P2Y_2$ receptors are G-protein coupled receptors, which stimulate phospholipase C and inositol triphosphate to induce an increase in intracellular Ca^{2+} and activation of

PKC. Agonists of the $P2Y_2$ receptor, such as ATP and UTP, can stimulate airway mucus secretion in humans and mammals (Kemp *et al* 2004, Murakami *et al* 2003).

1.3.6.5 Histaminergic receptor agonists

Histamine was one of the first mediators implicated in asthma. Whilst stimulating bronchoconstriction via the H_1 receptor, it also induces mucus secretion from goblet cells via the H_2 receptor (Tamaoki *et al* 1997). Additionally, despite mainly being mediated via the H_2 receptor, goblet cell mucus secretion may also be stimulated by histamine indirectly, via activation of H_1 receptors on cholinergic nerve terminals and subsequent release of the secretagogue, acetylchloine (ACh) (Takeyama *et al* 1996).

1.3.6.6 Th2-derived cytokines

Production of Th2 cells is associated with increased inflammation and mucus hypersecretion (Cohn *et al* 2002). Th2-derived cytokines including IL-4, IL-5, IL-9 and IL-13 have all been implicated in mucus secretion (Lee *et al* 1997, Temann *et al* 1997, Temann *et al* 1998, Cohn *et al* 2002). IL-13 especially, appears to be critically important in stimulating mucus secretion in allergic responses (Cohn *et al* 2002).

1.3.6.7 Inflammatory mediators

Several mediators important in inflammatory responses have been shown to be potent secretagogues in both human cell lines and in animal models. Eicosanoids such as leukotrienes and prostaglandins have been shown to increase goblet cell associated mucus secretion in guinea pigs and rats respectively (Hoffstein *et al* 1990, Takahashi *et al* 1999). The cytokine, tumour necrosis factor-α (TNF-α), can induce mucus secretion in the human airway (Lora *et al* 2005) and platelet activating factor (PAF) has been shown to stimulate mucus secretion in human airways (Goswami *et al* 1989). Lundgren *et al* (1991) have demonstrated mucus secretion by eosinophil cationic protein (ECP). Finally, neutrophil-derived enzymes such as human neutrophil elastase (HNE) and cathepsin G can induce airway submucosal gland or goblet cell mucus secretion (Nadel 1991, Park *et al* 2005)

1.3.6.8 Additional stimuli

Oxidative stress and cigarette smoke can also induce goblet cell mucin secretion (Bowler and Crapo 2002, Kuo *et al* 1992). Smoke-induced goblet cell-associated mucus secretion is likely to be mediated via irritant receptors and sensory efferent nerve activation (Rogers 2000).

1.3.7 REMOVAL OF MUCUS FROM THE LUNGS

Mucus is removed form the airways either by mucociliary clearance, which involves the cilia-facilitated movement of mucus to the mouth to be expectorated or swallowed or by cough, which dislodges mucus from the airways.

1.3.7.1 Mucociliary clearance (MCC)

Cilia are hair-like projections, present on the majority of epithelial cells, which waft and beat thousands of times per minute to transport mucus out of the airways. Mucus is transported by MCC at a rate of about 10mm/min in the larger airways and about 1mm/min in the smaller airways, resulting in the efficient removal of materials present in the lungs within 24 hours (Godwin 2002). Effective MCC is dependent on several factors. These include the quantity of mucus and the physical properties of mucus, such as its viscosity, elasticity and adhesivity (Mason 2005), which are determined by the relative proportion of mucins present in mucus. MCC is also dependent on functioning cilia and efficient cilia beat frequency (CBF) (Donno 2000). In disease, ciliary beat frequency can be decreased (Rossman *et al* 1983). Additionally, the maintenance of the correct sol layer is vital for efficient mucociliary clearance. The sol layer of the respiratory tract liquid maintains an optimum distance between the mucus layer and the epithelium and provides cilia with suitable conditions in which to beat (Tarran 2004). If the volume of sol layer is reduced, the overlying mucus layer can surround cilia and adhere to cell-surface tethered mucins, greatly reducing the efficiency of cilia movement and mucus clearance (Knowles and Boucher 2002).

The volume of the sol layer can increase or decrease depending on conditions in the airways. The airway epithelium acts as a regulator of ASL volume by secreting or absorbing ions from or into the ASL. Subsequent osmotic movement of water alters the

volume of the respiratory tract liquid layer (Tarran 2004). Although the exact ion channels responsible for this are unknown, several ion channels have been identified. These include the cystic fibrosis transmembrane conductance regulator (CFTR), the calcium activated Cl channel (CaCC) (Tarran *et al* 2002) and the epithelial sodium channel (ENaC) (Donaldson *et al* 2002).

1.3.7.2 Cough

Cough is an additional method of clearing mucus from the airways, which is dependent on the effectiveness of MCC, the height of the sol layer and mucus viscosity. Effective mucus clearance via cough is impaired by both a reduction in sol volume and an increase in mucus viscosity, due to adhesion of cell-tethered mucins and gel forming mucins (Knowles and Boucher 2002).

1.3.8 MUCUS HYPERSECRETION IN DISEASE

As discussed earlier, in diseased airways several pathophysiological changes in the airways can result in elevation of luminal airway mucus, which can be detrimental to health (Jackson 2001). For example, goblet cell metaplasia (phenotypic conversion of epithelial cells into goblet cells), submucosal gland hypertrophy, upregulation of MUC gene expression, goblet cell-associated mucus secretion and submucosal gland-associated mucus secretion can all be stimulated or upregulated, resulting in mucus plugging and airway obstruction. Mucus plugging and airway obstruction is associated with increased frequency of respiratory infections (Prescott *et al* 1995), probably due to the bacterial adhesion properties of mucin oligosaccharides (Vishwanath and Ramphal 1984), and increased airway inflammation. This can contribute to a reduction in lung function (Vestbo *et al* 1996) and an increased risk of hospitalisation and morbidity (Aikawa 1992). Mucus hypersecretion is an important characteristic of chronic inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF).

1.3.8.1 Asthma

Asthma is associated with increases in the number of goblet cells both in the larger airways, which are frequented with goblet cells, and in the smaller airways, which are usually absent of goblet cells. Asthma is also associated with changes in the structure of cilia microtubules and a reduction in the number of ciliated cells, due to goblet cell metaplasia and epithelial shedding. This can contribute to a reduction in ciliary beat frequency and a reduction in MCC (Donno *et al* 2000).

The expression of MUC genes differ in asthma compared to the normal airway (Ordonez *et al* 2001). In asthma, MUC5AC is the major gene upregulated in the airways (60% increased compared to normal airways) (Ordonez *et al* 2001). MUC2 and MUC4 levels were also increased. Mucins present in diseased airways of horses may possess different oligosaccharides to mucins in healthy lungs (Jefcoat *et al* 2001), resulting in increased mucus viscoelasticity and mucus accumulation. Sheehan *et al* (1995) demonstrated different cross-linking, size, acidity and appearance of asthmatic mucus, implying an abnormality in the assembly process of mucus in asthma.

1.3.8.2 Chronic Obstructive Pulmonary Disease (COPD)

COPD is a chronic disease of the airways and is the worlds estimated 5th largest cause of death (World Health Organisation 2007). The precise cause of COPD is unknown but is aggravated by smoking, viral infections, bacterial infections and genetic factors. It is characterised by limited airflow, difficulty in breathing and increased risk of infection, resulting from airway inflammation and airway remodelling. Pathophysiological changes during COPD include alveolar damage, bronchiolar damage, bronchial smooth muscle thickening in small airways, bronchoconstriction, submucosal gland hypertrophy with an increase in the mucous cell: serous cell ratio; goblet cell metaplasia, and inflammatory cell influx including CD8+ lymphocytes, macrophages and neutrophils (reviewed by Jeffery 2001).

However, mucus pathology differs in COPD and asthma (discussed by Rogers 2003). Unlike in asthma, in which the major upregulated mucin gene is MUC5AC, the MUC5B gene is the major gene that is upregulated in COPD (Caramori *et al* 2004). COPD is also associated with increased mucus viscosity, but is not associated with goblet cell tethering, which occurs in asthma, due to cleavage of the goblet cell-mucus

interaction by neutrophilic proteases (Rogers 2003). Furthermore COPD mucins are less acidic compared to asthma (Rogers 2003).

1.3.8.3 Cystic fibrosis (CF)

CF affects 70000 children and adults worldwide and is a major cause of premature death in sufferers (Cystic Fibrosis Foundation 2007). It is a genetic recessive disorder, resulting from a mutation in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (Roomans 2002). The CFTR is a membrane protein present in the epithelia, sweat glands, pancreas, intestine, liver and genito-urinary system. Principally, the CFTR acts as a cAMP regulated chloride channel, but can also act as a regulator of other channels such as the epithelial sodium channel and an ATP channel and/or regulator of ATP release (Stutts *et al* 1995). 1000 mutations of the CFTR gene exists and make up 4 classes: Class I.) CFTR protein production is decreased due to premature termination of the CFTR mRNA translation in the nucleus; Class II.) The protein is degraded within the ER; Class III.) The protein is positioned correctly in the membrane but does not respond properly to regulatory signals; Class IV.) The protein is positioned correctly in the membrane but possesses faulty transport properties (Boas and McColley 1997). There are several symptoms of cystic fibrosis, including salty sweat, pancreatic damage, excessive mucus production and lung damage. In the CF airways abnormal ion secretions due to non-functional CFTR leads to reduced airway fluid secretion and ultimately increased mucus viscosity. This contributes to airway mucus accumulation, mucus plugging and bacterial infection. Like asthma, it is the MUC5AC that is upregulated in the CF lung as a result of goblet cell metaplasia (Groneberg *et al* 2002).

1.3.9 MUCUS AND LUNG FUNCTION RESPONSES

Asthma is associated with significant reductions in lung function, resulting in increased risk of hospitalisation and mortality. However, asthma is a complex disease, characterised by bronchoconstriction, oedema, mucus accumulation, inflammation and airway remodelling. Due to the complex nature of asthma, it is difficult to determine the relative contribution of mucus airway accumulation and plugging to the decline in lung function revealed in asthmatic patients. In the past, mucus was considered an innocent

bystander of respiratory disease (Melton 2002). However, epidemiological studies suggest that mucus hypersecretion can have a dramatic effect on lung function, may lead to a loss of disease control and increased risk of mortality (Vestbo 2003). As a result of these findings, there has been intense interest in the impact that mucus hypersecretion and resulting airway obstruction has in respiratory diseases such as asthma, COPD and CF.

1.4 TREATMENT OF MUCUS HYPERSECRETION

To date there is no single class of compound that can effectively abolish mucus hypersecretion in inflammatory diseases of the airways such as asthma. However, several compounds may promote mucus clearance from the airways or potentially inhibit the production of mucin and therefore be used as pharmocotherapies for mucus hypersecretion. Such compounds target mucus hypersecretion at multiple levels, via several different mechanisms and may be categorised accordingly. Currently used therapies and potential compounds for mucus hypersecretion have been summarised by Rogers (2004) and are discussed below.

1.4.1 INFLAMMATION

Asthma is characterised by airway inflammation, mediated by numerous inflammatory cells and mediators. As discussed earlier multiple inflammatory mediators contribute to the mucus hypersecretory phenotype by stimulating goblet cell metaplasia, mucus production and mucus secretion. Compounds that inhibit inflammation via the inhibition of inflammatory mediators therefore have a beneficial effect on mucus hypersecretion. These include glucocorticoids, non-steroidal anti-inflammatories and PDE_4 inhibitors (Rogers 2004). Additionally, the COX-2 inhibitor NS398 blocked mucin production in NCI-H292 cells (Kim *et al* 2002), whilst an inhibitor of the EGFR TK inhibited goblet cell-associated mucin production in a rat asthma model (Takeyama *et al* 1999).

1.4.2 NERVE ACTIVATION

As previously discussed, goblet cell and submucosal gland mucus secretion may be stimulated via autonomic nerve activation (Section 1.3.6.5, 1.3.6.6 and 1.3.6.7). Airway mucus secretion is neuronally mediated via the M3 receptor. Anticholinergics, which are prescribed as bronchodilators for the treatment of COPD, appear to also reduce M3 receptor-mediated mucus secretion (Rogers 2000). The irritant fibres and C-fibres of the sensory afferent nervous system can respond to stimuli in the airways to increase mucus secretion. Opioids depress nerve activity, including neural responses in the airways such as mucus secretion e.g. in the guinea pig, morphine inhibits opioid receptors on sensory efferent fibres, thus inhibiting neurogenic mucus secretion (Kuo *et al* 1992). The NK_1

receptor appears to mediate mucus secretion (Ramnarine *et al* 1994) and an inhibitor of the tachykinin NK_1 receptor, MEN11467, has inhibited mucus secretion in ovalbumin (OA) challenged ferrets (Khan *et al* 2001). Therefore, the use of inhibitors of neurogenic inflammation may potentially have a beneficial effect on mucus hypersecretion in disease and therefore be a possible therapeutic option.

1.4.3 MUCUS SECRETION

Inhibition of secretagogues is an important pharmacological target for the treatment of mucus hypersecretion. As discussed earlier, inflammatory mediators released during the inflammatory response including PAF, ECP and neutrophil proteases such as HNE (batimastat), cathepsin G and protease 3 all stimulate mucus secretion and therefore have been identified as potential therapeutic targets for mucus hypersecretion. NPC15669 (a drug that inhibits leukocyte recruitment) and ICI 200355 (a neutrophil elastase inhibitor) can inhibit goblet cell degranulation (Rogers 2004). Additionally, infliximab (TNF-α), gefitinib (EGF), apafant (PAF), batimastat (HNE), montelukast (CysLT1 receptor antagonist), icatibant (bradykinin B_2 receptor antagonist), as well as potential inhibitors of the $P2Y_2$ receptor may all reduce mucus secretion and airway mucus accumulation (Rogers 2004).

Furthermore, the exocytosis of goblet cell-associated mucin is dependent on MARCKs, which facilitates intracellular movement of the mucin granule and docking and fusion of the mucin granule with the goblet cell membrane is likely to involve Munc18 homolog. Therefore, both MARCKs and Munc18 may be targeted to reduce exocytosis of mucin granule and mucin secretion. Singer *et al* (2004) have synthesised a peptide (MANS peptide) corresponding to the N-terminal domain of MARCKs and demonstrated that intratracheal instillation of the peptide inhibited mucin secretion in mice.

1.4.4 MUC GENE UPREGULATION

Multiple stimuli appears to induce MUC gene expression and mucin production via the EGFR (Mason 2005). Therefore inhibition of signalling via the EGFR by EGFR TK inhibitors may significantly reduce upregulation of MUC gene expression and mucus production. Additionally, expression of the Ca^{2+} activated Cl^- channel (hCLCA1) is associated with increased production of gel-forming mucin. Niflumic acid (NFA), an

inhibitor of hCLCA1 significantly reduced goblet cell metaplasia (Zhou *et al* 2002). Furthermore MAPK and PI-3 kinase appear to be important intracellular signalling pathways involved in MUC gene upregulation. In OA sensitised and challenged guinea pigs the phosphoinositide 3-kinase inhibitor, LY294002, significantly reduced goblet cell-associated mucin accumulation (Duan *et al* 2005) and in OA sensitised and OA challenged mice, nebulised exposure of a p38 MAPK antisense oligonucleotide (p38a-ASO) inhibited mucus production (Duan *et al* 2005).

1.4.5 GOBLET CELL METAPLASIA

Activation of the EGFR on airway epithelial cells inhibit apoptosis, thus allowing IL-13-stimulated differentiation of ciliated cells into goblet cells (Tyner *at al* 2006) suggesting that inhibition of the EGFR or IL-13 may reduce goblet cell metaplasia. Additionally, pro-apoptotic factors or anti-anti-apoptotic factors may also inhibit goblet cell metaplasia.

1.4.6 MUCUS ACCUMULATION

Mucolytic agents and related compounds are used for the treatment of mucus hypersecretion, particularly in chronic bronchitis (discussed by Yuta and Baraniuk 2005). Mucolytic agents, such as N-acetylcysteine reduce mucus viscosity by breaking disulphide linkages between gel-forming mucins, thus allowing mucus to be more effectively removed from the airways. Expectorants such as guanfenesin act as an irritant to increase airway liquid secretion and stimulate cough, therefore aiding mucus clearance form the airways (Yuta and Baraniuk 2005). However, the therapeutic benefit of these compounds has been widely scrutinised. Several trials (Mucomyst, Apothecon, Plainsboro, NJ) have shown no definite benefit by N-acetylcysteine and there is little evidence in support of the use of guanfenesin (Kellerman 2002).

Additionally, stimulation of MCC may increase mucus clearance from the airways and benefit patients with a mucus hypersecretory phenotype. Drugs that may stimulate ciliary beat frequency include long lasting β agonists (salmeterol) (Piatti *et al* 2004), methylxanthines (Wanner 1985) and $P2Y_2$ receptor-specific agonists such as INS316 and INS365 (Inspire Pharmaceutics 2007).

1.5 GUINEA PIG MODELS OF ASTHMA

In the clinical setting, asthmatic individuals demonstrate bronchoconstriction, inflammation and airway hyperresposniveness following natural exposure to inhaled allergens. The sensitised guinea pig exhibits both early and late phase bronchoconstrictor responses, inflammation, demonstrated by inflammatory cell recruitment to the lungs and hyperreactivity to inhaled histamine following exposure to inhaled antigens such as ovalbumin (Smith and Broadley 2007) and is therefore an excellent model of human asthma.

Technically, the use of guinea pigs as experimental models has several advantages over the use of other animal models. Guinea pigs are small, relatively inexpensive, easy to handle and easy to maintain. The docile nature of the guinea pig allows measurement of lung function responses in a conscious animal using plethysmography. This is non-invasive, and due to the lack of anaesthesia, reflex pathways that may affect lung function are not suppressed. This also means that lung function measurements can be repeated and obtained over extended periods of time in the same animal.

However, the physiology of a guinea pig lung differs from that of a human lung. Guinea pigs are obligatory nose breathers and so associating changes in lung function measurements solely to changes in the lower airways and not to changes in nasal resistance may present difficulties.

Furthermore, when utilising animal models for scientific research, it is essential to consider physiological species differences. In humans and higher mammals, submucosal glands are sparse in the lower airways (non-cartilaginous airways), but populate the trachea and larger airways (cartilaginous airways) (Goco *et al* 1963). However, mice, rats, guinea pigs and hamsters have few submucosal glands in the airways and only express submucosal glands in the upper portion of the trachea (Widdicombe *et al* 2001, Choi 2000).

In humans, goblet cells are dispersed throughout large (cartilaginous) and small (non-cartilaginous) airways (Cerkez *et al* 1986). However, whereas human airways possess both submucosal glands and goblet cells, the goblet cell, which is populated throughout the guinea pig airway, is the main source of mucus in the guinea pig (Jackson 2001).

Control of airways secretions, also appears to display significant species differences. Goblet cell secretion in humans and higher mammals is largely under non-neuronal control and submucosal gland secretion under neuronal control, whereas in rodents, which possess few submucosal glands, neuronal control of goblet cells is significant (Jackson 2001).

1.6 AIMS AND OBJECTIVES

The aim of my project was to identify potential changes in lung function responses in an animal model, displaying a mucus hypersecretory phenotype.

- To develop and optimise a guinea pig chronic OA model of asthma in sensitised guinea pigs that demonstrates early and late phase bronchoconstriction, cellular infiltration, airway hyperreactivity and goblet cell-associated mucin production (an important feature of human asthma not identified following acute OA exposure).
- To examine lung function responses in animals with a mucus hypersecretory phenotype to determine whether mucus hypersecretion was associated with a reduction in lung function.
- To analyse the effect of nebulised exposures of potential secretagogues, including purinergic receptor agonists (UTP, UDP and ATP), adenosine receptor agonists (adenosine) and histamine receptor agonists (histamine) on goblet cell-associated mucin secretion and lung function responses in chronically OA challenged guinea pigs.
- To identify the effect of secretagogue antagonists including suramin (P2 receptor antagonist) and ranitidine (H_2 receptor antagonist) on potential lung function responses and goblet cell mucin secretion following secretagogue exposure.
- To validate a Sandwich Enzyme Linked Lectin Assay (Sandwich ELLA) for the measurement of mucin in guinea pig BALF.
- To utilise the Sandwich ELLA for the measurement of mucin in guinea pig BALF in order to demonstrate possible alterations in mucus output in secretagogue-treated and secretagogue antagonist-treated guinea pigs.

CHAPTER 2

Methods

2.1 MATERIALS & EQUIPMENT

2.1.1 MATERIALS

All materials and reagents were supplied by Sigma unless otherwise stated.

2.1.2 EQUIPMENT

In-house built plethysmograph.

DeVilbiss Pulmostar Nebuliser, Sunrise Medical Ltd (Wollaston, UK)

RM machines PC PL466 486DX (Abingdon, UK) with Acqknowledge® software

Biopac® data acquisition system: Biopac System Inc Model MP100 linked to Biopac systems TCI 100 (analogue digital coverter), Biopac® systems Inc (Santa Barbara, USA).

Pressure transduces Pioden Type 1, Pioden Controls Ltd (Canterbury, UK).

Wax dispenser, Fisher (Loughborough, UK).

Cold plate, Fisher (Loughborough, UK).

Neubauer improved light haemocytometer, Supe-rior (Marienfeld, Germany).

Cytospin II, Shandon (Runcorn, UK).

Centrifuge, Mistral 3000 (Mistral, UK).

Leica microtome, Leica Microsystems (Wetzlar, Germany).

Camedia C4040ZOOM Digital camera, Olympus (London, UK)

BH-2 Olympia Microsope, Olympus (London, UK)

2.2 ANIMAL EXPERIMENTS

2.2.1 ANIMAL HUSBANDRY

In all studies male Dunkin-Hartley guinea pigs (supplied by Harlan, UK) weighing between 200-250g were used. Groups of six animals were held in grid-bottomed cages. The animals were allowed animal feed and drinking water ad libitum and supplied with cardboard tubes for environmental enrichment. The temperature was maintained at 20°C±2°C, 50% humidity and light/dark periods (12hrs) alternated. Animal experiments were carried out in accordance with the Animal Scientific Procedures Act 1986.

2.2.2 SENSITISATION

At least 7 days subsequent to delivery to the animal facility, animals were sensitised on days 1 and 5 with an intra-peritoneal (i.p.), bilateral injection of a suspension containing 100 μg of OA and 100 mg aluminium hydroxide. Aluminium hydroxide is an adjuvant, which increases the immune response.

2.2.3 INHALATION EXPOSURES

Following the sensitisation period, guinea pigs were exposed to an acute OA challenge or chronic OA challenge. For all challenges, a Wright nebuliser was used to supply air at a pressure of 20p.s.i and at a rate of 0.3ml/min into a stainless steel exposure chamber (40cm diameter, 15cm height). If any of the animals looked in distress, they were withdrawn from the chamber and exposure considered complete.

2.2.3.1 Acute OA challenge

14 days following sensitisation (day 15), animals were exposed to a nebulised solution of OA (0.01% for 1hr). Lung function measurements were taken prior to and subsequent to OA challenge at 0, 15, 30, 45 and 60mins, then hourly for 12hrs and at 24hrs.

2.2.3.2 Chronic ovalbumin challenges

14 days following sensitisation (day 15), animals were exposed to a single nebulised solution of low dose OA (0.01% for 1hr). Animals were subsequently exposed to a nebulised solution of low dose OA (0.01% for 1hr) or high dose OA (0.1% for 1hr) on days 17, 19, 21, 23, 25, 27, 29 and 31. Administration of the H_1 receptor antagonist mepyramine (30mg/kg) by bilateral intra-peritoneal (i.p.) injection was carried out 30mins prior to high dose OA exposures on days 17, 19, 21, 23, 25 and 27, in order to protect against fatal anaphylaxis. Chronic OA challenge protocols are detailed in Chapter 3.

2.2.3.3 Assessment for airway hyperreactivity (AHR).

A 3mM dose of inhaled histamine (20secs) induces a bronchoconstriction response in naïve guinea pigs. However, a 1mM dose of inhaled histamine (20secs) induces a threshold bronchoconstriction in untreated guinea pigs but a more substantial bronchoconstriction response in OA sensitised and OA challenged guinea pigs and is utilised as a measure of AHR. To measure AHR, guinea pigs were exposed to a single inhaled threshold dose of histamine 24hrs prior to and 24hrs subsequent to acute or chronic OA challenges. Guinea pigs were placed in a small animal restrainer consisting of high sides and a neck restrainer, which ensured secure positioning of the animal (Fig. 2.1). A Wright nebuliser was used to supply histamine solution (1mM for 20secs) at air pressure of 20p.s.i. and at a rate of 0.3ml/min via a narrow plastic tunnel with a small mouthpiece attached (sealed by means of a cut balloon). Lung function responses were measured prior to and subsequent to histamine exposure at 0, 5 and 10mins.

2.2.3.4 Box exposures to induce mucus secretion

Subsequent to acute OA challenge or chronic OA challenge, animals were exposed to a nebulised solution of histamine, UTP, UDP, ATP or 5'AMP to potentially induce goblet cell-associated mucus secretion. For all exposures, a Wright nebuliser was used to supply air at a pressure of 20p.s.i and at a rate of 0.3 ml/min into a sealed perspex chamber (15 x 15x 32cm).

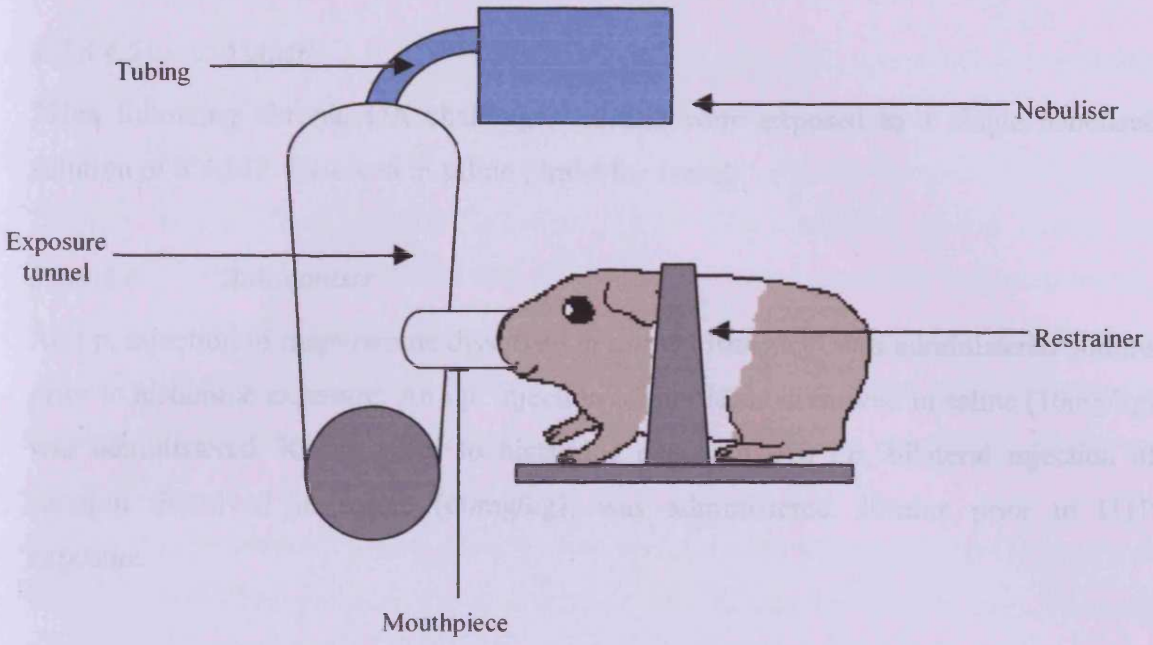


Figure 2.1. Schematic diagram identifying the restrainer and exposure tunnel used to expose guinea pigs to inhaled histamine (threshold dose).

2.2.3.4.1 Histamine

22hrs 30mins following chronic OA challenge, animals were exposed to a single nebulised solution of histamine dissolved in saline (10mM or 100mM for 30mins).

2.2.3.4.2 UTP

22hrs 45mins following chronic OA challenge, animals were exposed to a single nebulised solution of UTP dissolved in saline (1mM or 10mM for 15mins).

2.2.3.4.3 UDP

22hrs 45mins following chronic OA challenge, animals were exposed to a single nebulised solution of UDP dissolved in saline (1mM or 10mM for 15mins).

2.2.3.4.4 ATP

23hrs following chronic OA challenge, animals were to a single nebulised solution of ATP dissolved in saline (3mM for 1min).

2.2.3.4.5 5'AMP

23hrs following chronic OA challenge, animals were exposed to a single nebulised solution of 5'AMP dissolved in saline (3mM for 1min).

2.2.3.4.6 Antagonists

An i.p. injection of mepyramine dissolved in saline (30mg/kg) was administered 30mins prior to histamine exposure. An i.p. injection of ranitidine dissolved in saline (10mg/kg) was administered 30mins prior to histamine exposure. An i.p. bilateral injection of suramin dissolved in saline (60mg/kg), was administered 30mins prior to UTP exposure.

2.2.4 LUNG FUNCTION MEASUREMENTS

During respiration, differences in pressure between the mouth and alveoli (alveolar pressure difference) allow air to enter the lungs. Airway resistance (R_{aw}) is the air pressure difference between the alveoli and mouth divided by airflow (v). Alterations in alveolar pressure difference produces changes in the volume of air in the lungs, or thoracic gas volume (TGV), and this ultimately results in pressure changes within the constant volume plethysmograph. Plethysmography measures specific airway conductance (sG_{aw}), which is defined as the reciprocal of R_{aw} per unit TGV (Tattersfield and Keeping 1981) and is often used instead of R_{aw} in order to correct for TGV (Griffiths-Johnson *et al* 1998).

The plethysmograph is an airtight perspex box, which is accessed via a removable endplate. Four ports exit the box, one to record box pressure, two to measure airflow at the mask, and one to open the box to atmosphere. An animal restrainer, consisting of high sides and a neck restrainer, ensured secure positioning of the guinea pig to allow a perspex mask to be sealed to the snout by means of a cut balloon. Removal of the plethysmograph endplate permitted the guinea pig and restrainer to be slid into the plethysmograph, and replacement of the endplate provided an airtight environment to allow for lung function measurement (Fig. 2.2).

A UP1 and UP2 pressure transducer measured changes in respiratory flow and box pressure respectively. Box pressure was measured by one pressure transducer, one side of which is connected to the plethysmograph and the other side to the atmosphere. Flow

(v) was measured with a mesh pneumatograph (Mercury FIL) connected to a pressure transducer. The flow signals were passed through a computer system with a Biopac® data acquisition system and Acqknowledge® software (Biopac® systems Inc, Santa Barbara, USA) (Danahay and Broadley 1997). The computer system stored the respiratory flow and box pressure data as waveforms, which correlate with each breath. The recording period was 5secs, allowing for measurement of at least five breaths. Typical waveforms measured using the Acqknowledge® data acquisition pack, representing changes in box pressure and airflow in a conscious guinea pig, are shown in Fig. 2.3.

The resulting waveforms were differentiated and analysed by comparing the gradients of the flow and box pressure waves at a point were flow tends towards zero. For each breath sG_{aw} was calculated. sG_{aw} is a function of these parameters, correcting for ambient pressure and the weight of the animal (Toward and Broadley 2002). The theory behind the use of sG_{aw} to measure lung function is detailed in Appendix I. To reduce stress to the animal, the guinea pigs were familiarised with the apparatus daily for 3 days prior to the experiment.

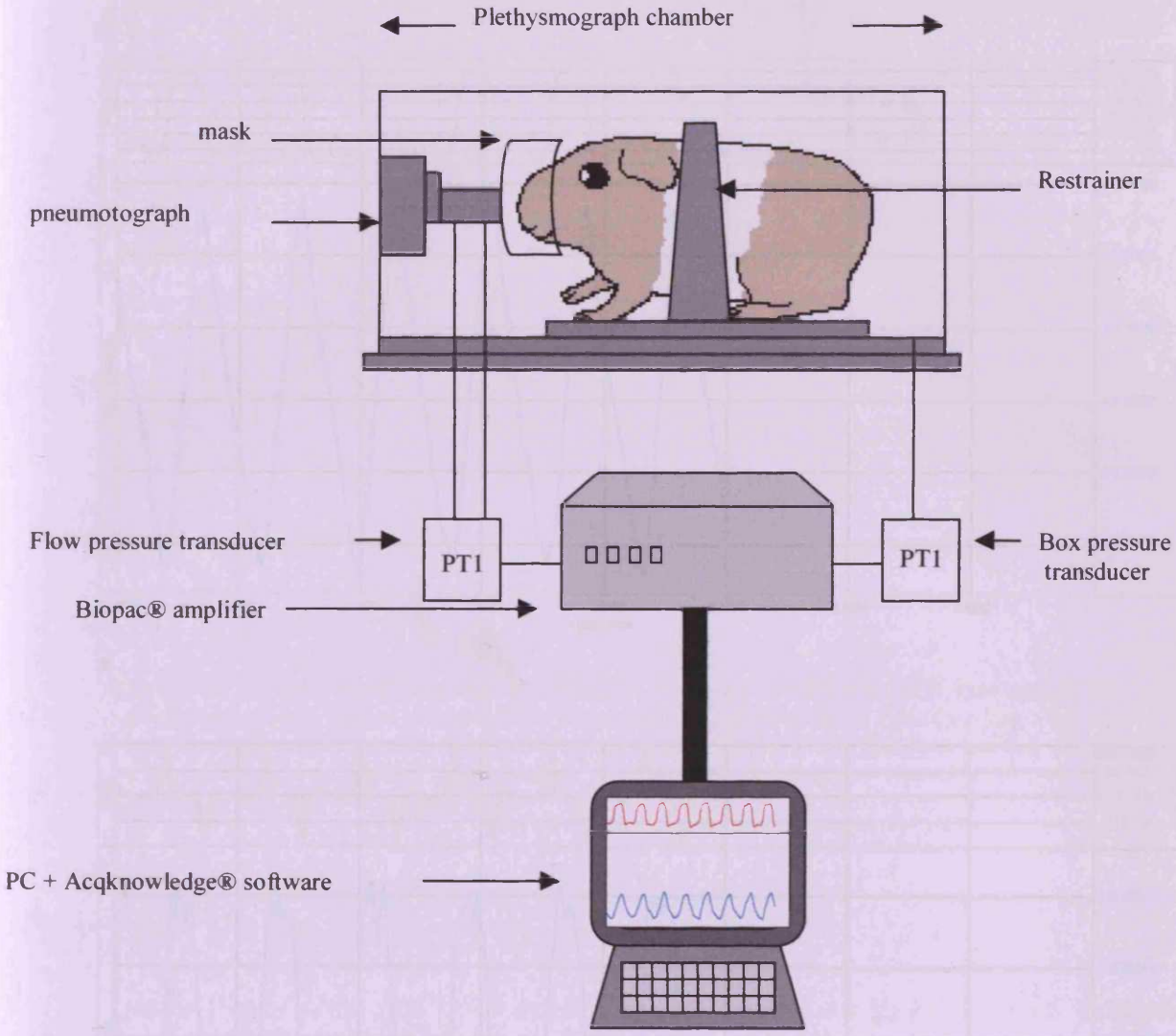
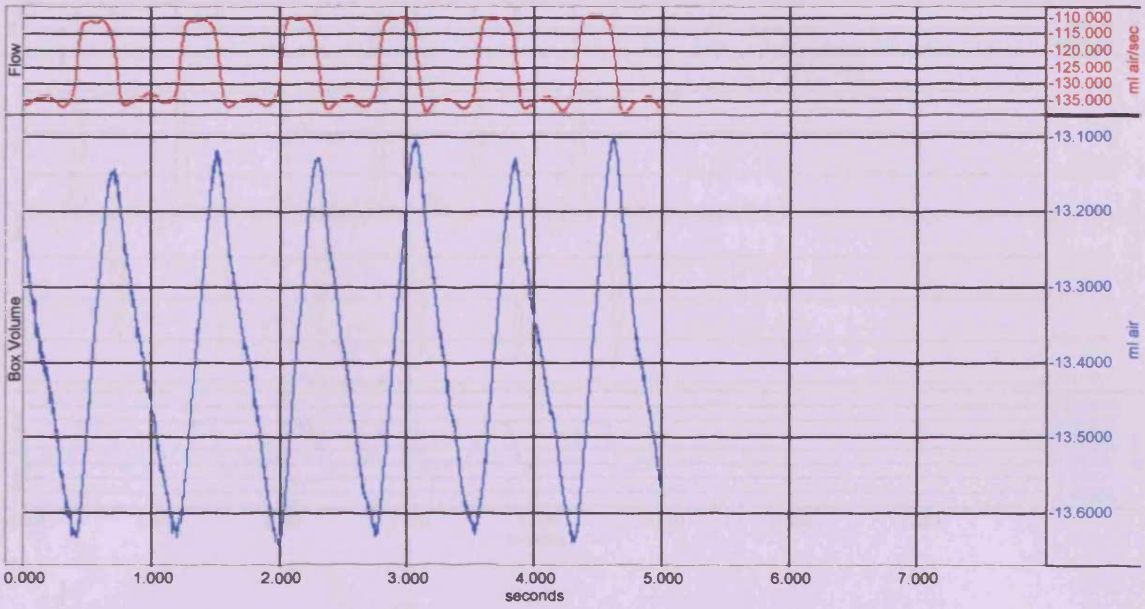


Figure 2.2. A schematic diagram identifying the plethysmograph and acquisition packs used to measure sG_{aw} in restrained, conscious guinea pigs


Flow
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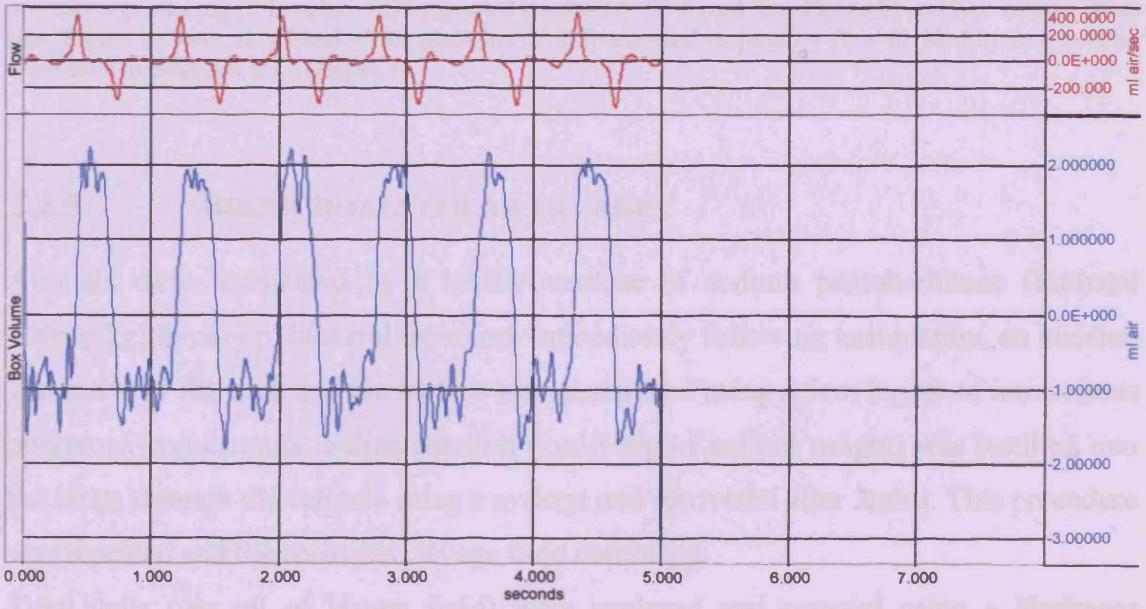
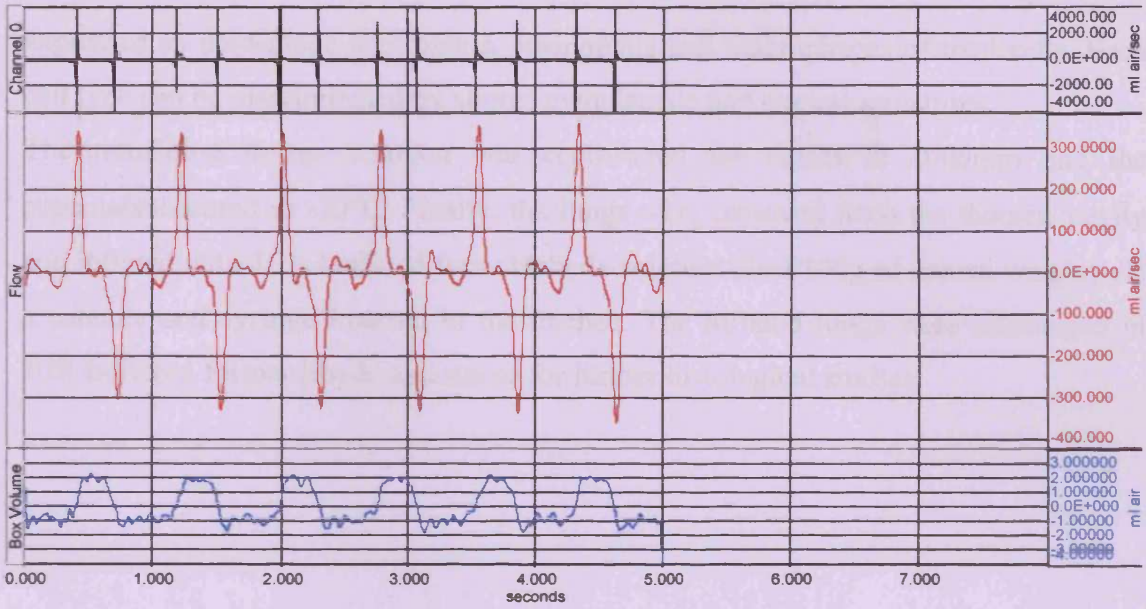
-115.000
-120.000
-125.000
-130.000
-135.000
ml air/sec
Box Volume
-13.1000
-13.2000
-13.3000
-13.4000
-13.5000
-13.6000
ml air
0.000
1.000
2.000
3.000
4.000
5.000
6.000
7.000
seconds

a


Flow
400.0000
200.0000
0.0E+000
-200.000
ml air/sec
Box Volume
2.000000
1.000000
0.0E+000
-1.00000
-2.00000
-3.00000
ml air
0.000
1.000
2.000
3.000
4.000
5.000
6.000
7.000
seconds

b



c

Figure 2.3. Typical baseline waveforms measured using the Acqknowledge® data acquisition pack representing changes in box pressure (blue line, Figure a) and respiratory flow (red line, Figure a) in a conscious guinea pig (6 breaths). Differentiated respiratory flow (red line, Figure b) and box volume (blue line, Figure b) were calculated. Peak gradients of differentiated respiratory flow divided by box volume were used to calculate sG_{aw} (Figure c).

2.2.5 BRONCHOALVEOLAR LAVAGE

Animals were terminated by a lethal overdose of sodium pentobarbitone (Euthatal 400mg/kg) by an i.p. bilateral injection. Immediately following termination, an incision was made in the neck and the trachea was cannulated using a 5cm length of intravenous polypropylene cannula. Saline solution (1ml/100g of animal weight) was instilled into the lungs through the cannula using a syringe and recovered after 3mins. This procedure was repeated and the recovered lavage fluid combined.

Total cells (per ml of lavage fluid) were analysed and counted using a Neubauer haemocytometer (Supe-rior, Marienfeld, Germany). 100µl of the lavage solution was centrifuged for 7mins at 1000rpm onto a glass slide using a cytospin (Cytospin II, Shandon, Runcorn, UK). Subsequent to air-drying, the slide was stained using 1.5% Leishman's solution in 100% methanol and allowed to re-dry. A minimum of 200 cells were counted to determine differential cell counts. Differential cell counts were

expressed as percentage neutrophils, eosinophils and macrophages of total cells. Each cell type can be discriminated by altered cytoplasmic and nuclear structures.

The remaining lavage solution was centrifuged for 6mins at 2000rpm and the supernatant stored at -20°C. Finally, the lungs were removed from the thoracic cavity and inflated with 10% buffered formaldehyde solution (2ml/100g of animal weight) via a cannula and syringe inserted in the trachea. The inflated lungs were submerged in 10% buffered formaldehyde and stored for further histological studies.

2.3 HISTOLOGICAL ANALYSIS OF GUINEA PIG LUNGS

To enable sectioning of very thin slices of animal tissue, guinea pig lungs were processed and set into a wax solid phase. Subsequent staining of tissue sections allows the identification of particular cellular or tissue components.

2.3.1 HISTOLOGICAL PROCESSING

A 3-5mm thick portion of the superior lobe of the left lung was sliced tangentially 1mm below the bronchus. The lung slices were then held in Surgipath histology cassettes and processed through the following solutions:

1. 50% aqueous industrial methylated spirit (IMS, Fischer) for 1hr
2. 70% aqueous IMS for 1hr
3. 90% aqueous IMS for 1hr
4. 100% IMS for 1hr 30mins
5. 100% IMS for 1hr 30mins
6. 100% IMS for 1hr 30mins
7. 50% IMS: 50% chloroform (BDH) for 1 hr
8. 100% chloroform for 2 hrs
9. 100% chloroform for 2 hrs
10. Molten paraffin wax (58°C, Surgipath) for 2 hrs
11. Molten paraffin wax for 2 hrs
12. Molten paraffin wax for 2 hrs

2.3.2 EMBEDDING OF GUINEA PIG LEFT LUNG SLICES

The processed lung slices were placed in metal histology moulds (Sigma), immersed in molten paraffin wax, and subsequently allowed to set on cold plates.

2.3.3 SECTIONING OF PARAFFIN-EMBEDDED GUINEA PIG LEFT LUNG SLICES

Sections (3μm) of paraffin-embedded guinea pig lung samples were sliced using a Leica microtome and fixed on a glass slide. Slides were then allowed to dry overnight in a 37°C oven.

2.3.4 HISTOLOGICAL STAINING OF PARAFFIN SECTIONS OF GUINEA PIG LEFT LUNG: AB/PAS STAINING

Slides were stained with alcian blue/periodic acid Schiff (AB/PAS) and Mayers haemolum using the following protocol:

1. Paraffin sections of guinea pig left lung were dewaxed in xylene for 5mins and taken through the following graded IMS concentrations:
   a. 100% IMS for 5mins
   b. 100% IMS for 5mins
   c. 90% IMS for 5mins
   d. 70% IMS for 5mins
2. Sections were washed in distilled water for 5mins.
3. Sections were immersed in 1% AB dissolved in 3% aqueous acetic acid (pH 2.5) for 5mins.
4. Sections were rinsed in running tap water for 5mins.
5. Sections were immersed in periodic acid (0.5%) for 5mins.
6. Sections were rinsed in running tap water for 5mins.
7. Sections were washed in distilled water for 5mins.
8. Sections were immersed in Schiffs reagent (Surgipath) for 10mins
9. Sections were rinsed in running tap water for 10mins
10. Sections were dipped in Mayers haemolum for 20secs
11. Sections were rinsed in running tap water for 5mins.
12. Sections were taken through the following graded IMS concentrations:
    a. 70% IMS for 5mins
    b. 90% IMS for 5mins
    c. 100% IMS for 5mins

d. 100% IMS for 5mins

13. Sections were cleared in xylene for 5mins

2.3.5 ANALYSIS OF ALCIAN BLUE/PERIODIC ACID SCHIFF (AB/PAS) STAINED SECTIONS

AB/PAS staining is the mainstay protocol used for the positive identification of mucin in the airways of several laboratory animals (Sueyashi *et al* 2004, Komori *et al* 2001). The different binding profiles of AB and PAS allow the beneficial differentiation between neutral mucins that stain magenta (from PAS) and acidic mucins that stain blue (from AB). Sections (3μm) of the upper quarter of guinea pig left lung were stained with AB/PAS and Mayers haemolum. The area of purple/magenta stain in the bronchial epithelium of guinea pig lung sections identified goblet cell associated mucin. All bronchioles in each section were photographed using a digital camera (Olympus, London, UK) and microscope (Olympus, London, UK). See Fig. 2.4a for a typical section of lung taken from a sensitised and OA challenged guinea pig. The images were then analysed using SigmaScan Image Analysis program (Systat Software Inc., London, UK). A draw tool was used to manually trace the periphery of the bronchiolar epithelium and the resulting area (expressed as numbers of pixels) determined the area of epithelium (Fig. 2.4b). Quantification of total AB/PAS stained epithelial area (also expressed as number of pixels) was determined by utilisation of a SigmaScan magenta/purple colour threshold measurement (Fig. 2.4c). The area of AB/PAS-stain positive cells was expressed as the % of the total epithelial area. This was calculated for each bronchiole and mean values calculated.

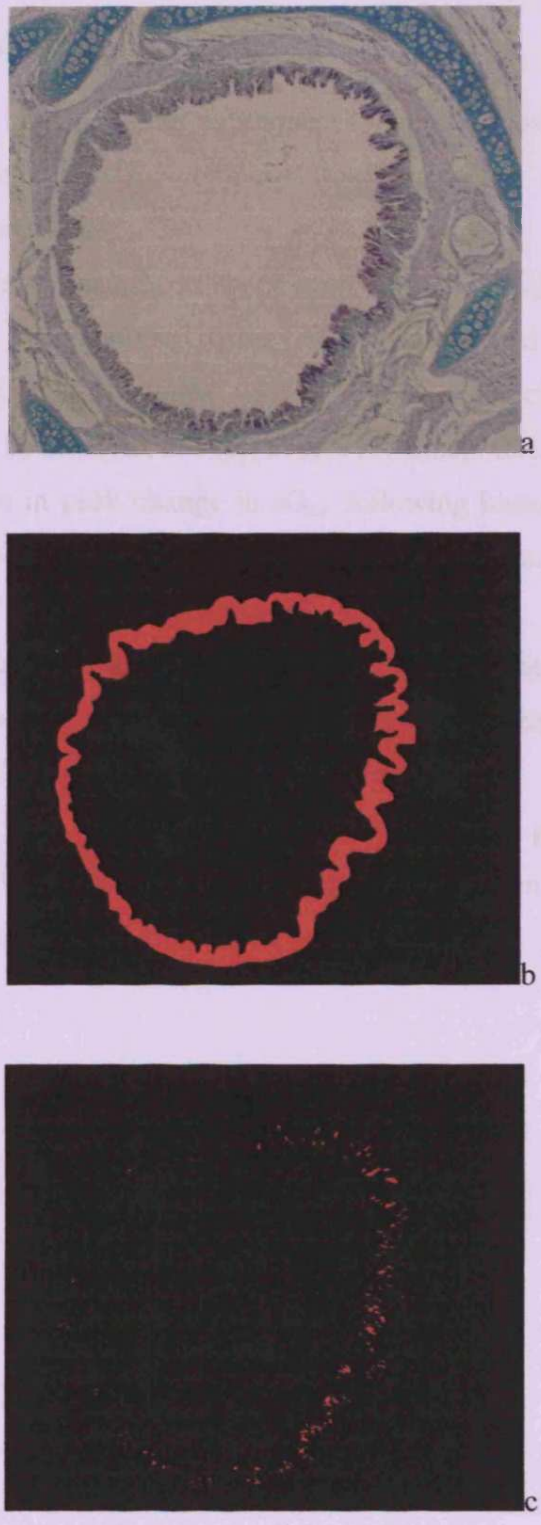


Figure 2.4 Guinea pigs were sensitised and chronically challenged with OA. Paraffin section (3μm) of guinea pig left lung were taken and stained with AB/PAS. Photograph a) demonstrates a typical AB/PAS stained bronchiole. Image b) represents a trace of the bronchiole epithelial area, which can subsequently be used to determine total epithelial area (expressed as number of pixels). Image c) represents the AB/PAS-positive area (expressed as number of pixels) of the bronchiole epithelium, determined by SigmaScan Image Analysis (USA) colour threshold measurement.

2.3.6 STATISTICAL ANALYSIS

Time course changes in sG_{aw} values subsequent to nebulised exposures were expressed as a percentage change in sG_{aw} compared to the baseline sG_{aw} values (measured immediately prior to exposure).

Statistical significance of changes in specific airway conductance (sG_{aw}) compared to baseline sG_{aw} values was analysed using ANOVA followed by Student's t-test on specific timepoints. If multiple groups were compared, statistical analysis was assessed by ANOVA followed by Bonferroni's test at specific timepoints.

Significant differences in peak change in sG_{aw} following histamine challenge prior to and subsequent to challenges were assessed by a Students t-test. Where multiple groups were assessed, ANOVA followed by Bonferroni test was used.

Significant differences in $\log_{10}$ values of total and differential cell counts were compared using a Student's t-test. When differences between multiple groups were assessed, ANOVA followed by Bonferroni's test was used.

Statistical differences in % AB/PAS-positive epithelial area in guinea pig airways were assessed using ANOVA followed by Student's t-test. When multiple groups were compared, ANOVA followed by Bonferroni's test was used.

CHAPTER 3

Development of a chronic asthma model and tolerance to chronic allergen challenges

3.1 INTRODUCTION

3.1.1 ANIMAL MODELS OF ASTHMA

In a genetically predisposed individual the clinical symptoms of human asthma are stimulated following a single inhalation of allergen. This phenomenon has led to the development of laboratory models of asthma aimed to represent the clinical setting. Following an acute exposure to a nebulised antigen, such as ovalbumin (OA), sensitised guinea pigs exhibit multiple human asthmatic responses such as early and late phase bronchoconstrictor responses, inflammation, demonstrated by inflammatory cell recruitment to the lungs, and hyperreactivity to inhaled histamine (Smith and Broadley 2007). The acute OA model of asthma has been extensively studied and has been vital in our ongoing understanding of the mechanisms behind the initiation of an asthmatic response. However, although in the clinical setting asthma is commonly identified as recurrent inflammatory response, resulting from repeated exposure to inhaled allergen, there has been significantly less investigation into the allergic responses of the airways following chronic allergen challenges.

3.1.2 ASTHMA AND THE IMMUNE RESPONSE

The human respiratory tract mucosa is repeatedly exposed to a myriad of foreign particles that are inhaled from the air (Banchereau and Stenmann 1998). The immune system of a non-atopic individual is able to distinguish between pathogenic antigens such as virus and bacteria, which are capable of damage to the host, and innocuous environmental antigens, which are not pathogenic. This is an extremely complex immunological function, given the extensive number and diversity of foreign particles that interact with the airway mucosa. Failure of the immune system to regulate the appropriate responses to innocuous antigens can result in allergic sensitisation; this is observed in the asthmatic individual (Palliser *et al* 1998).

The process of allergic sensitisation involves antigen presenting cell (APC)-facilitated proliferation of naïve T cells into Th2 cells. Activated Th2 cells subsequently produce a specific profile of cytokines, which induce antigen-specific IgE production by B cells and the expression of IgE-specific, high affinity Fc epsilonRI receptors on the surface of

mast cells. Subsequently, circulating IgE antibodies target and fix mast cell surface IgE receptors in the airways. This mechanism allows immediate recognition of the same antigen following re-exposure and rapid initiation of an allergic response. Antigen binding to mast cell fixed IgE results in mast cell activation and subsequent mast cell degranulation. This leads to the release of multiple pro-inflammatory mediators and the initiation of an inflammatory cascade, mediated by numerous cell types and mediators, which manifests clinically as an asthmatic response (Pradalier *et al* 1993).

3.1.3 MUCUS ACCUMULATION AND ANIMAL MODELS OF CHRONIC ASTHMA

The development of a chronic mucus hypersecretory phenotype is an important pathological feature of respiratory diseases such as asthma. Excessive mucus production and secretion in the airways can lead to mucus plugging, which may contribute to airway obstruction and exacerbation of compromised airways (Jackson *et al* 2001). In an asthmatic airway, the physiology of the bronchiolar epithelium is altered and the airway secretory potential is increased via enlarged submucosal glands and an increased number of surface epithelial goblet cells (Benayoun *et al* 2003, Ordonez *et al* 2001). Both increased epithelial stored mucin and increased mucus secretion has been demonstrated following nebulised OA exposure in sensitised animals (Blyth *et al* 1996, Agusti *et al* 1998). This study will investigate the effects of chronic OA challenges on epithelial goblet cell-associated mucin accumulation in order to develop a chronic model of asthma, characterised by increased goblet cell-associated mucin accumulation as well as EAR, LAR, AHR and inflammatory cell influx into the airways. A chronic OA model demonstrating increased stored mucin may prove to be a clinically relevant model of asthma and subsequently provide a valuable tool to allow the study of the development of a mucus hypersecretory phenotype. However, the induction of tolerance has previously been revealed following chronic allergen challenge in several animal models (Schramm *et al* 2004) and this must therefore be considered in the chronically OA challenged guinea pig.

3.1.4 MECHANISMS OF TOLERANCE AND ASTHMA

A consequence of chronic allergen exposure in sensitised animals is the development of tolerance to an allergen that has previously stimulated an allergic response. This was revealed in a murine model of asthma following repeated exposure of OA (Schramm *et al* 2004) and may also translate to humans. For example, studies across Europe and in Australia have indicated that exposure to cat dander in childhood may reduce the prevalence of asthma later in adult life (de Meer *et al* 2004, Roost *et al* 1999). Additionally, the use of repeated allergen exposure has been considered a therapeutic strategy for desensitising patients with allergic disease for over 100 years (Noon *et al* 1911).

The precise mechanisms involved in the development of tolerance following inhaled allergen exposure, and therefore delivery of allergen via the respiratory mucosa, are unclear. However, there is a considerable body of research attempting to elucidate the mechanisms behind the development of oral tolerance and delivery of allergen via the gut mucosal surface. Suggested mechanisms of oral tolerance include immune deviation, clonal deletion, anergy, and active suppression. Although there has been significantly less focus on the development of respiratory tolerance, these mechanisms have also been implicated in the development of respiratory tolerance (Lowrey *et al* 1998) and are discussed below.

3.1.4.1 Physical mechanisms of tolerance

Both structural and immunological mechanisms contribute to the airway defence system and protect the respiratory system against inhaled allergen. Physical barriers such as epithelial tight junctions and secreted mucus prevent the passage of allergen across the airway epithelium and subsequent initiation of an immune response. Additionally, the mucociliary transport system and cough reflex contribute to the removal of allergen from the airways and lung secretions in the upper and lower airways demonstrate antimicrobial properties.

3.1.4.2 Pulmonary alveolar macrophages

The pulmonary alveolar macrophage may play a role in the development of unresponsiveness to allergen. They are the main phagocytes within the airways, but

despite this have extremely poor capacity to present antigen to T cells (Holt 1986). The activation of T cells cannot be stimulated simply by whole antigen. Rather, it requires processing of antigen by an antigen presenting cell (APC) and the presentation of peptide to the T cell in the presence of co-stimulatory signals. However alveolar macrophages may lack co-stimulatory molecules on their cell surface, resulting in allergen-induced suppression of T cells (Chelen *et al* 1995), exhibited as anergy or clonal deletion (see below).

3.1.4.3 Clonal deletion and anergy

In order to protect against an autoimmune response, the body's immune system has the ability to eliminate T cells that can recognise self-proteins via both anergy and clonal deletion (Romagnani 2006). Clonal deletion is described as activation-induced apoptosis of peptide specific CD4+ T cells and anergy is described as functional inactivation of peptide specific CD4+ T cells (Palliser *et al* 1998). Anergy is maintained by the persistence of peptide *in vivo* and requires interaction between allergen and the T cell receptor (TCR) during T cell activation (Palliser *et al* 1998). Activation of T cells by antigen requires 2 signals: 1) TCR recognition of peptide-MHC molecules on the surface of APCs and 2) a co-stimulatory, non-specific interaction. The interaction between peptide and the TCR is stabilised by engagement of the TCR-associated CD3 molecule with the peptide, which allows initiation of T cell activation. The non-specific co-stimulatory signal involves interaction between CD molecules on the T cell surface, which are expressed following peptide recognition and one of the B7 family of molecules present on the APC. This initiates a cascade of signalling events that induces T cell proliferation (Vigouroux *et al* 2004).

The precise mechanism of anergy is not fully understood. The initial proposed mechanism for the induction of anergy involved activation of T cells via their TCR in the absence of co-stimulation (Lowrey *et al* 1998). Subsequent hypotheses include: 1) the conformational model suggests that an incorrect conformation of peptide in the TCR can result in the transmission of a negative signal, which induces inactivation (Janeway 1995). 2) the kinetic discrimination model suggests that reduced TCR and peptide interaction time induces inactivation (Rabinowitz *et al* 1996). 3) the most recent proposal, the dose-dependent model, suggests that T cell activation requires a threshold

number of interactions with peptide (more than 100), whilst fewer interactions (between 1 and 10) induces T cell inactivation/anergy (Korb *et al* 1999). It is possible that both clonal deletion and anergy play a role in the development of respiratory tolerance.

3.1.4.4 Regulation by T cells

Friedman and Weiner (1994) suggest that anergy is usually favoured by high dose antigen and that low doses of antigen by the oral route may lead to suppression of CD4+ T cells. Regulation or suppression of an immune response can be mediated by the production of antigen-specific regulatory T cells (T_{reg}), formerly known as suppressor cells, via the secretion of inhibitory cytokines. T_{reg} cells are not easily identified, but can be distinguished according to their distinctive cytokine profile (Vigouroux *et al* 2004). The Th3 cells, a subset of CD4+ T cells, secrete large amounts of TGF-β and smaller quantities of IL-10 and IL-4 (Chen *et al* 1994), whilst the Tr1 cells secrete large amounts of IL-10 and smaller quantities of IL-2, IL-4 and TGF-β (Groux *et al* 1997).

The mechanism behind the production and activation of T_{reg} cells is unclear. However, negative signalling during T cell activation, positive signalling (for example the ICOS (Akbari *et al* 2002) or NOTCH pathways (Hoyne *et al* 2000)) during T cell activation, and the influence of cytokines have all been implicated.

Several groups have demonstrated inhibitory effects of the T_{reg}-derived cytokines, IL-10 and TGF-β, on the Th2 immune response. Both T_{reg} inhibitory cytokines, IL-10 and TGF-β, inhibit the growth of T cells by preventing the proliferation of naïve T cells into Th2 cells and subsequently downregulate the Th2 immune response (Akdis and Blaser 2001, Tiemesson *et al* 2003). Akbari *et al* (2002) demonstrated that T_{reg} cells display potent inhibitory activity when adoptively transferred to sensitised mice and this is dependent on IL-10 production and ICOS-ICOS interactions. IL-10 and TGF-β, in addition to suppression of IgE production, also appear to directly or indirectly suppress inflammatory cells, such as mast cells, basophils and eosinophils. Furthermore, T_{reg} cells may also play a role in the induction of clonal deletion and anergy of CD4+T cells following allergen challenge (Akbari *et al* 2001).

3.1.4.5 Immune deviation

In an atopic individual, allergen exposure in early infancy leads to priming of Th2 CD4+ T cells, which produce IgE and results in allergic sensitisation. Alternatively in a non-atopic individual, rather than a simple lack of recognition of allergen, a Th1 immune response is mounted against allergen. This is associated with the production of cytokines such as IL-2, IFN γ and TNF-α/β, which initiate various effects including activation of macrophages, differentiation of CD8+ T cells into cytotoxic killer cells, inhibition of Th2 cell functions, limitation of B cells and IgG2a antibody production. On continued allergen exposure in an atopic individual, CD4+ T cells can switch their pattern of cytokine production from a Th2 to a Th1 phenotype in a process called immune deviation. In mice studies, an IgE (Th2) response was demonstrated following low level nebulised OA exposure (Holt *et al* 1981). However, continuation of exposure resulted in alleviation of the Th2 response and subsequent unresponsiveness to antigen, possibly due to immune shift from a Th2 to a Th1 response.

A subset of CD8+ T cells appears to play an important role in maintaining tolerance to allergic response. McMenamin and Holt (1993) demonstrated that nebulised OA exposure in rats resulted in the production of a population of CD8+ T cells which had the capacity to transfer suppression of IgE synthesis to naïve animals. It was subsequently revealed that this subset of CD8+ T cells possessed a γδ TCR, instead of the common αβ TCR (McMenamin *et al* 1994). These cells are now considered important regulator cells, inducing deviation from a Th2 to a Th1 immune response. The γδ CD8+ T cells block IgE synthesis and airway hyperreactivity (AHR) (McMenamin *et al* 1994), possibly via the secretion of IFN-γ (McMenamin and Holt 1993) and additional cytokines/mediators (Macaubas *et al* 2003).

However, further complexity results from conflicting findings regarding γδ CD8+ T cells. Increased γδ CD8+ T cell numbers were observed in the airways of asthmatic patients following allergen challenge and this was associated with increased Th2 cytokine levels (Krug *et al* 2001). γδ CD8+ T cells have also been shown to promote allergic airway inflammation (Zuany-Amorim *et al* 1998) and IFN-γ may contribute to the severity of disease. Additionally, several research groups failed to identify a role for CD8+ T cells or IFN-γ in the development of tolerance following allergen exposure (Seymour *et al* 1998). The investigations which revealed that γδ CD8+ T cells mediate

both Th1 and Th2-like responses has led to the proposed existence of 3 different subsets of γδ CD8+ T cells, each exerting different effects on the immune system.

In this chapter, the development of a chronic asthma model will involve repeated OA challenges in the sensitised guinea pig. Tolerance to repeated OA challenges has previously been demonstrated in mice (Schramm *et al* 2004) and may be revealed in the chronically OA challenged guinea pig. It will therefore be an important consideration when developing a chronic asthma model in the guinea pig.

3.2 AIMS AND OBJECTIVES

HYPOTHESIS. *Chronic allergen challenge in the sensitised guinea pig will induce goblet cell metaplasia and increase epithelial stored mucus.*

3.2.1 AIM

The aim of this chapter was to develop a chronic OA model of asthma in sensitised guinea pigs. In addition to early and late phase bronchoconstriction, cellular infiltration and airway hyperreactivity, the model must demonstrate increased goblet cell-associated mucin production, an important feature of human asthma not identified following acute OA exposure. In subsequent chapters this model will be utilised to study the effect of mucus secretagogues in the airways of guinea pigs with increased goblet cell-associated mucin content.

3.2.2 OBJECTIVES

- To ascertain the effect of repeated low-dose OA challenges on the parameters measured in acutely challenged guinea pigs i.e. EAR, LAR, AHR, total and differential BALF cell counts.
- To investigate the effect of repeated high-dose OA challenges on EAR, LAR, AHR, total and differential BALF cell counts
- To identify and quantify the AB/PAS-positive area of the bronchiolar epithelium in paraffin sections of OA challenged guinea pig airways using histological methods and SigmaScan Image Analysis computer software.
- To optimise a chronic OA model that reveals EAR, LAR, AHR, inflammatory cellular infiltration to the airways and increased goblet cell-associated mucin production.

3.3 METHODS

Groups of 6 male Dunkin-Hartley guinea pigs (supplied by Harlan, UK) weighing between 200-250g were used for all protocols.

3.3.1 SENSITISATION

Animals were sensitised on days 1 and 5 with an intra-peritoneal (i.p), bilateral injection of a suspension containing 100μg of OA and 100mg aluminium hydroxide.

3.3.2 NEBULISED OVALBUMIN CHALLENGES

14 days subsequent to the sensitisation period (day 15), guinea pigs were challenged with either an acute low dose OA challenge (0.01% for 1hr) or one of 4 chronic OA challenges, described in Table 3.1. For control exposures, guinea pigs were exposed to either an acute saline exposure (1hr) on day 15 or a chronic saline exposure, described in Table 3.1. Only one chronic vehicle challenge was carried out, in order to limit the number of animals required. For all challenges, a Wright nebuliser was used to supply air at a pressure of 20p.s.i. and at a rate of 0.3ml/min into a sealed stainless steel exposure chamber (40cm diameter, 15cm height). If any animal appeared in distress, the animal was removed from the exposure chamber and the challenge considered complete.

3.3.3 LUNG FUNCTION MEASUREMENTS

Whole body plethysmography was used to measure sG_{aw} as previously described (Section 2.2.4). Lung function measurements were taken before challenge and subsequent to challenge at 0, 15, 30, 45 and 60mins, then hourly up to 12hrs and at 24hrs. Values were expressed as % change in sG_{aw} from initial baseline sG_{aw} (measured immediately prior to OA challenge). In order to analyse late phase asthmatic responses, which occur at various timepoints depending on each animal, the peak falls in sG_{aw} between 6 and 12hrs were averaged.

Exposure Day	Chronic OA challenge 1	Chronic OA challenge 2	Chronic OA challenge 3	Chronic OA challenge 4	Chronic vehicle challenge
Day 15	0.01%	0.01%	0.01%	0.01%	saline
Day 17	0.01%	0.01%	0.1% M	0.1% M	saline M
Day 19	0.01%	0.01%	0.1% M	0.1% M	saline M
Day 21	0.01%	0.01%	0.1% M	0.1% M	saline M
Day 23	0.01%	0.01%	0.1% M	0.1% M	saline M
Day 25	0.01%	0.01%	0.1% M	0.1% M	saline M
Day 27	N/A	0.01%	0.1% M	0.1% M	saline M
Day 29	N/A	0.01%	0.01%	0.1%	saline
Day 31	N/A	N/A	0.1%	N/A	N/A

Table 3.1. Chronic OA challenge protocols (1-4). Sensitised guinea pigs were exposed to one of four chronic OA challenges subsequent to sensitisation on days 15 to 31. Each exposure period was 1hr. The dose of nebulised OA used is shown. M= mepyramine (30mg/kg) dissolved in saline and administered by i.p. bilateral injection 30mins prior to OA exposure.

3.3.4 ASSESSMENT FOR AIRWAY HYPERREACTIVITY

To assess AHR (as previously described in Section 2.2.3.3), animals were exposed to a single nose-only nebulised solution of histamine (1mM for 20secs) 24hrs prior to OA challenge and 24hrs subsequent to acute OA challenge or the last OA challenge of chronic OA challenges 1-4. A Wright nebuliser was used to supply air at a pressure of 20p.s.i and at a rate of 0.3ml/min. Lung function responses were measured immediately prior to histamine exposure and 0, 5 and 10mins subsequent to histamine exposure.

3.3.5 TOTAL AND DIFFERENTIAL CELL COUNTS

24hrs subsequent to acute OA challenge or chronic OA challenge animals were terminated by a lethal overdose of sodium pentobarbitone and lungs lavaged. Total cells and differential cell counts (per ml of lavage fluid) were determined using a Neubauer

haemocytometer and cytospin spears respectively, as previously described (Section 2.2.5).

3.3.6 HISTOLOGICAL ANALYSIS OF GUINEA PIG LUNGS

3-5mm tangentially sliced portions of lung were processed into wax blocks, sectioned (3μm) using a Leica microtome and fixed onto glass slides. Slides were stained with AB/PAS and Mayers haemolum and each bronchiole analysed for the mean % of AB/PAS-positive area of the bronchiolar epithelium. This was calculated for each bronchiole and mean values calculated. Detailed methodology is described in Chapter 2.

3.4 RESULTS

3.4.1 ACUTE LOW-DOSE OA CHALLENGE IN SENSITISED GUINEA PIGS

The effect of an acute low dose OA challenge in sensitised guinea pigs on lung function responses, AHR, BALF total and differential cell numbers and goblet cell associated mucin accumulation was assessed.

3.4.1.1 Effect of an acute low-dose OA challenge on lung function

Fig. 3.1 represents the mean time course for changes in specific airway conductance (sG_{aw}) following a single nebulised OA challenge (0.01% for 1hr) in sensitised guinea pigs. Exposure to nebulised OA resulted in an EAR, identified as an immediate reduction in sG_{aw} (-75.0%). sG_{aw} values were recovered to baseline at 6hrs following OA challenge, and this was proceeded by a LAR, demonstrated by a significant reduction in sG_{aw} (-23.3%) between 7 and 10hrs subsequent to OA challenge. No changes in sG_{aw} were revealed in sensitised guinea pigs following a single vehicle (saline) exposure (1hr).

3.4.1.2 Effect of an acute low-dose OA challenge on AHR

Fig. 3.2 represents changes in sG_{aw}, compared to baseline, following exposure to nose-only inhaled histamine (1mM for 20secs) 24hrs prior to and 24hrs subsequent to an acute low dose OA challenge. At this dose, histamine failed to initiate changes in sG_{aw} 24hrs prior to OA challenge. However, an inhaled histamine exposure revealed significant AHR, identified as an immediate reduction in sG_{aw}, 24hrs following OA challenge. No response to histamine was observed in sensitised guinea pigs, prior to or subsequent to a single nebulised vehicle (saline) exposure (1hr).

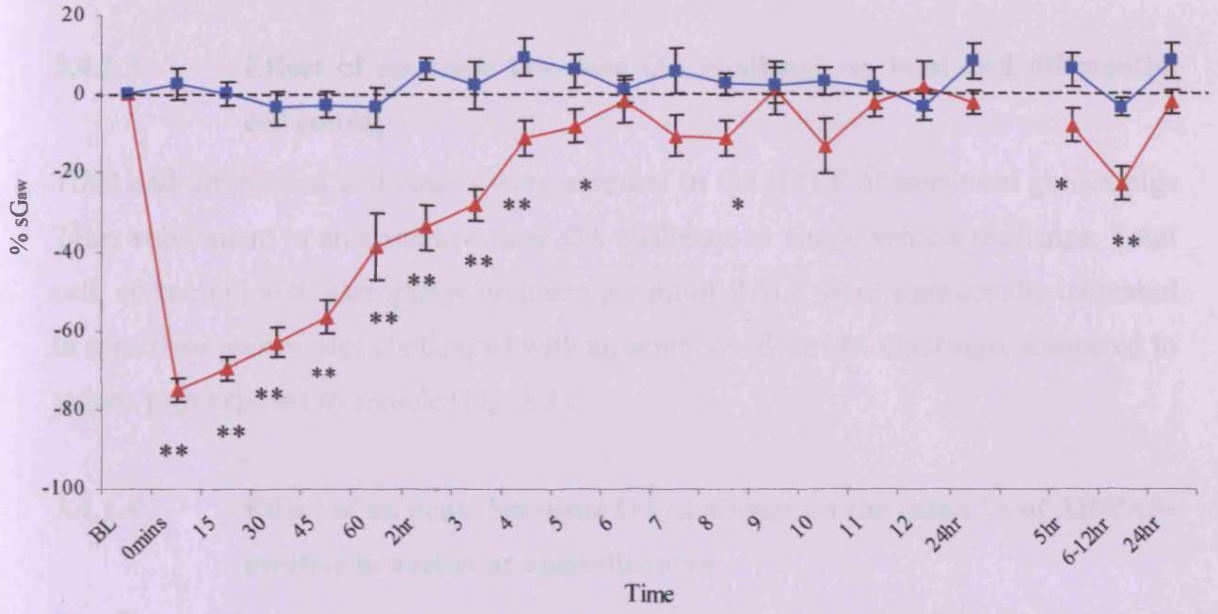


Figure 3.1. The effect of an acute nebulised low-dose OA challenge (0.01%/1hr) (▲) or a single nebulised vehicle exposure (1hr) (■) on lung function measurements in sensitised guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw}. The mean fall in sG_{aw} between 6 and 12 hrs after OA challenge is also shown. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different from vehicle.

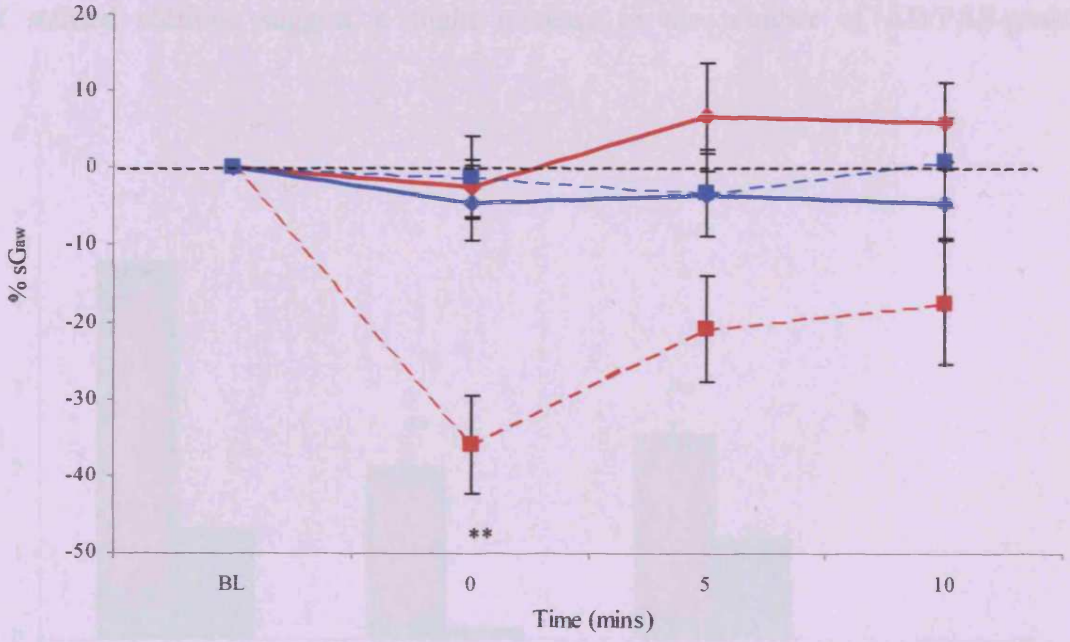


Figure 3.2. The effect of an inhaled nose-only nebulised exposure of histamine (1mM/20secs) 24hrs prior to (◆) and 24hrs subsequent to (■) an acute low dose OA challenge (0.01%/1hr) on lung function measurements in sensitised guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. Lung function measurements following a single vehicle exposure (20secs) prior to (◆) and subsequent to (■) an acute low dose OA challenge are also shown. n=6. ** ($p<0.01$) significantly different from vehicle.

3.4.1.3 Effect of an acute low-dose OA challenge on total and differential cell counts

Total and differential cell counts were assessed in the BALF of sensitised guinea pigs 24hrs subsequent to an acute low-dose OA challenge or single vehicle challenge. Total cell, eosinophil and macrophage numbers per ml of BALF were significantly increased in sensitised guinea pigs challenged with an acute low dose OA challenge, compared to guinea pigs exposed to vehicle (Fig. 3.3).

3.4.1.4 Effect of an acute low-dose OA challenge on the mean % of AB/PAS-positive bronchiolar epithelial area

Paraffin sections (3μm) of guinea pig left lung were stained with AB/PAS and Mayers haemolum and the mean % of AB/PAS-positive bronchiolar epithelial area calculated (as described in Chapter 2). Fig. 3.4a and b represent typical AB/PAS-stained sections (3 μm) of tangentially sliced guinea pig left lung removed from acutely saline challenged guinea pigs and acutely OA challenged guinea pigs respectively. Inspection of stained sections suggest a slight increase in the number of AB/PAS-positive

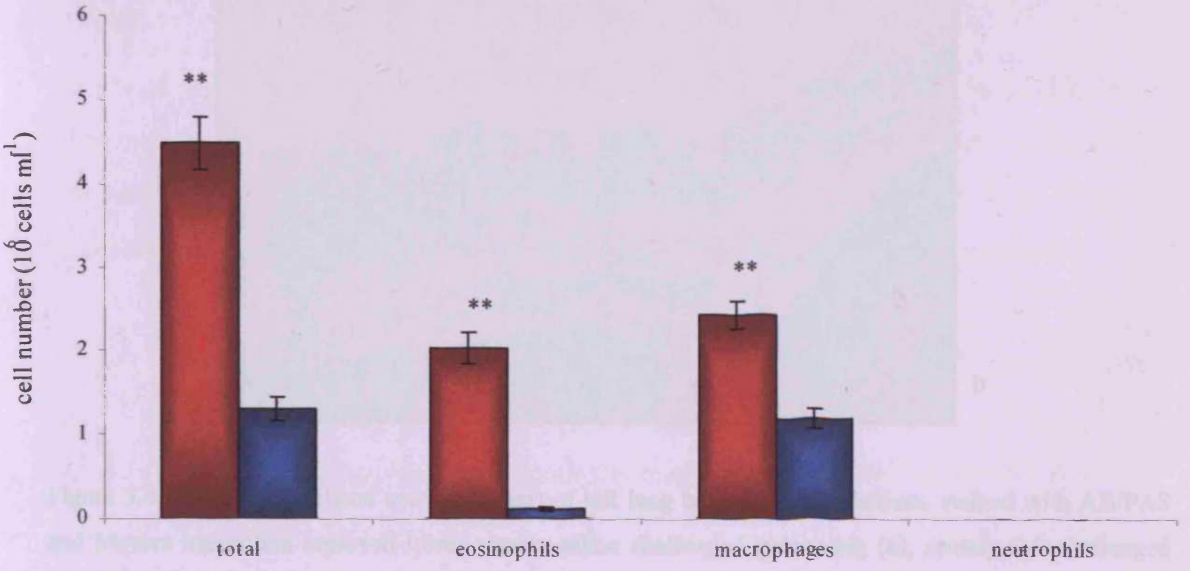


Figure 3.3. The effect of an acute low-dose OA exposure (0.01%/1hr) (■), or vehicle exposure (■) on total and differential cell counts in the BALF of sensitised guinea pigs 24hrs subsequent to OA challenge. Results are expressed as mean ± s.e.m. cell numbers per ml of BALF. ** ($p<0.01$) significantly different from vehicle.

epithelial cells in the airways of acutely saline challenged guinea pigs, compared to acutely OA challenged animals (Fig. 3.4), although statistical analysis revealed no significant difference (Fig. 3.5).

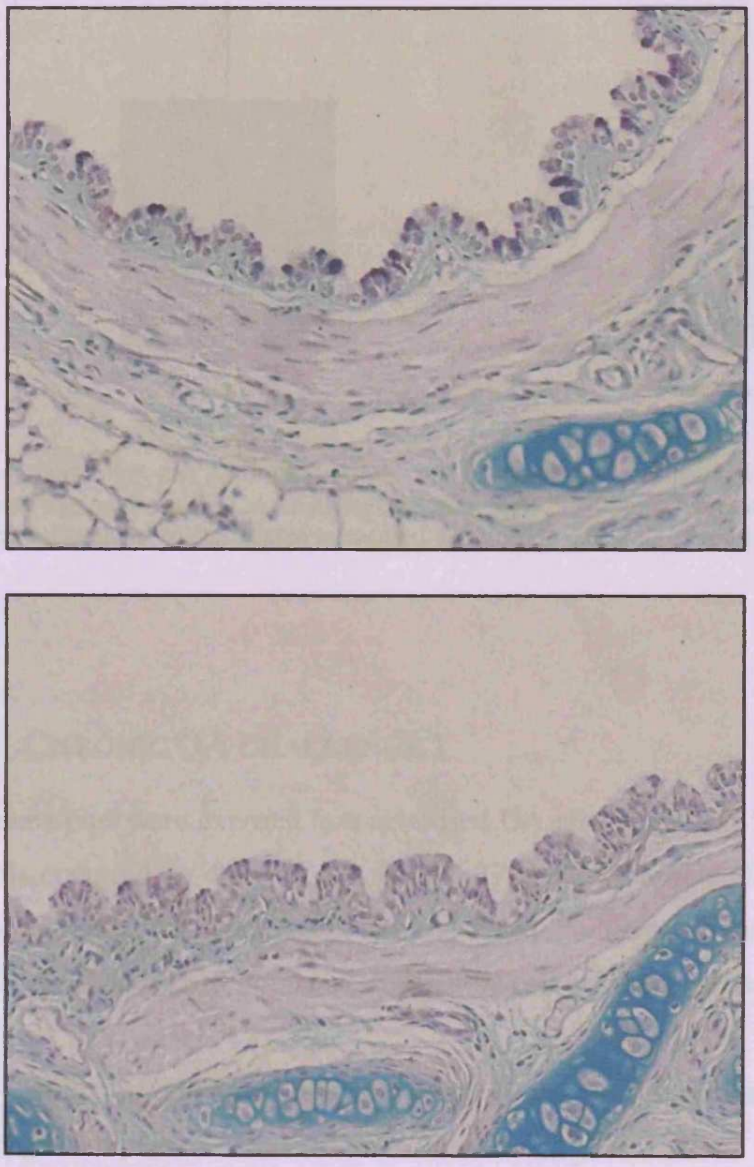


Figure 3.4. Tangentially sliced sections (3μm) of left lung bronchiolar epithelium, stained with AB/PAS and Mayers haemolum removed from: acutely saline challenged guinea pig (a); acutely OA challenged guinea pig (b).

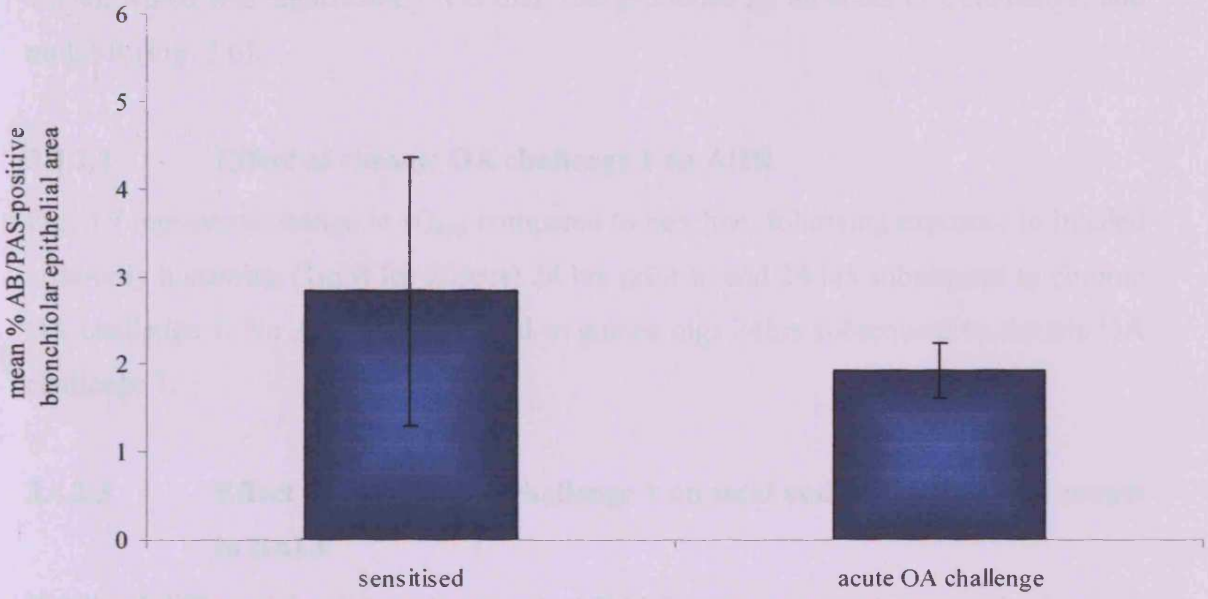


Figure 3.5. Comparison of the AB/PAS-positive area of bronchiolar epithelium in acutely saline challenged guinea pigs and acutely OA challenged guinea pigs. Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of tangentially sliced guinea pig left lung. n=6.

3.4.2 CHRONIC OA CHALLENGE 1

Sensitised guinea pigs were exposed to a nebulised OA challenge (0.01% for 1hr) on 6 occasions. This occurred on days 15, 17, 19, 21, 23 and 25 (chronic OA challenge 1). The effect of chronic OA challenge 1 in sensitised guinea pigs on lung function measurements, AHR and BALF total and differential cell numbers was assessed.

3.4.2.1 Effect of chronic OA challenge 1 on lung function in sensitised guinea pigs

Fig. 3.6 represents the mean time course for changes in sG_{aw} following the final OA challenge of chronic OA challenge 1 (day 25). Baseline sG_{aw} values were measured immediately prior to nebulised OA on day 25 and sG_{aw} values measured up to 24hrs subsequent to OA challenge. Lung function responses following chronic OA challenge 1 differed significantly to lung function responses following an acute OA challenge. Nebulised low dose OA (0.01% for 1hr) on day 25 produced a significant EAR (-34.3 ±

7.3%), which was significantly less than that produced by an acute OA challenge, and no LAR (Fig. 3.6).

3.4.2.2 Effect of chronic OA challenge 1 on AHR

Fig. 3.7 represents change in sG_{aw}, compared to baseline, following exposure to inhaled nose-only histamine (1mM for 20secs) 24 hrs prior to and 24 hrs subsequent to chronic OA challenge 1. No AHR was revealed in guinea pigs 24hrs subsequent to chronic OA challenge 1.

3.4.2.3 Effect of chronic OA challenge 1 on total and differential cell counts in BALF

Total and differential cell counts per ml of BALF were measured in sensitised guinea pigs, 24hrs subsequent to an acute low-dose OA challenge or chronic OA challenge 1. There was no significant difference in total cell, eosinophil or macrophage numbers per ml of BALF following chronic OA challenge 1 compared to acute OA challenge (Fig. 3.8).

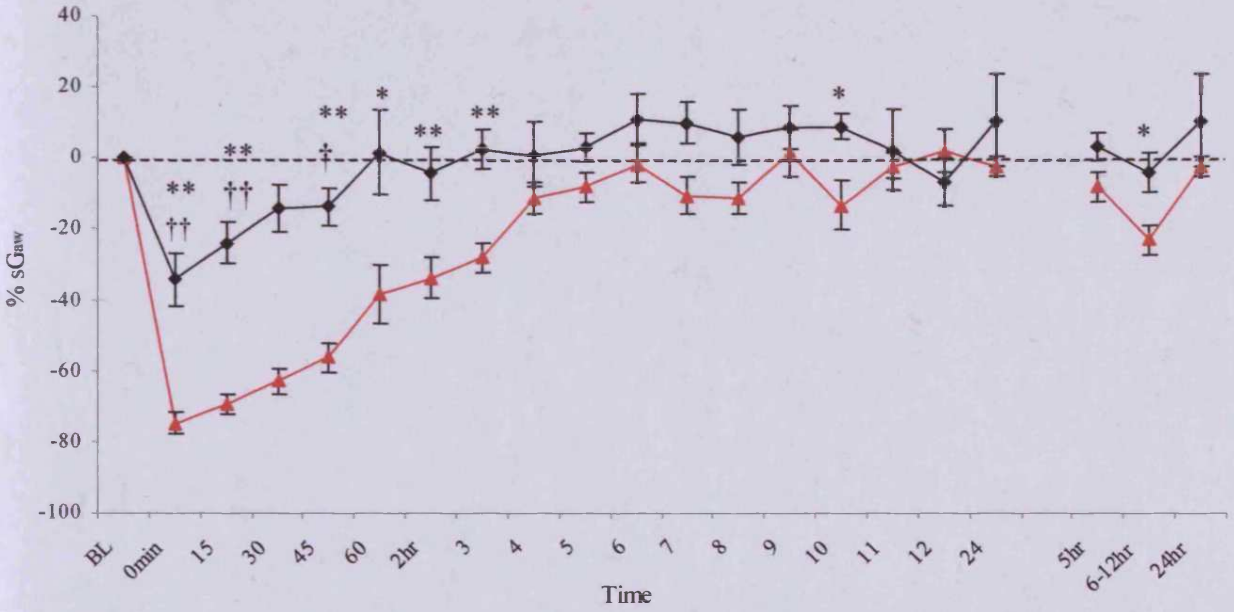


Figure 3.6. The effect of an acute low-dose OA challenge (0.01%/1hr) (▲) or the final low-dose OA challenge (0.01%/1hr) of chronic OA challenge 1 (day 25) (◆) on lung function measurements in sensitised guinea pigs. Each point represents the mean ± sem change in sG_{aw} compared to baseline sG_{aw} values. The mean fall in sG_{aw} between 6 and 12hrs after OA challenge is shown. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different from acute OA challenge. † ($p<0.05$) †† ($p<0.01$) significantly different from baseline.

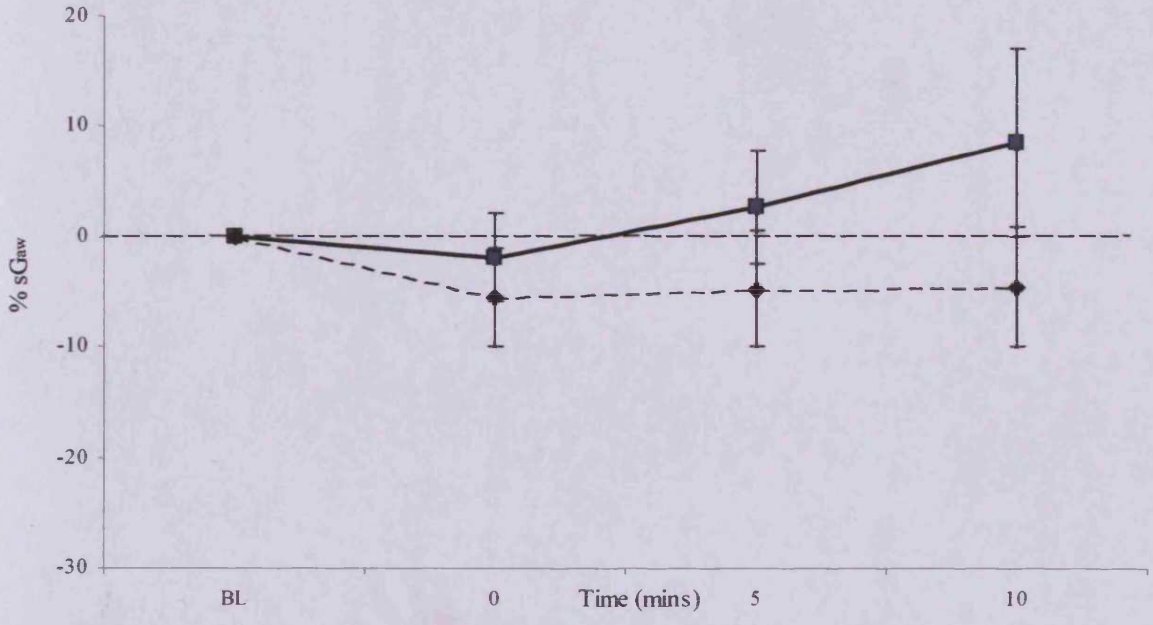


Figure 3.7. The effect of an inhaled nose-only nebulised exposure of histamine (1mM/20secs) 24hrs prior to (■) and 24hrs subsequent to (◆) chronic OA exposure 1 on lung function measurements in sensitised guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6.

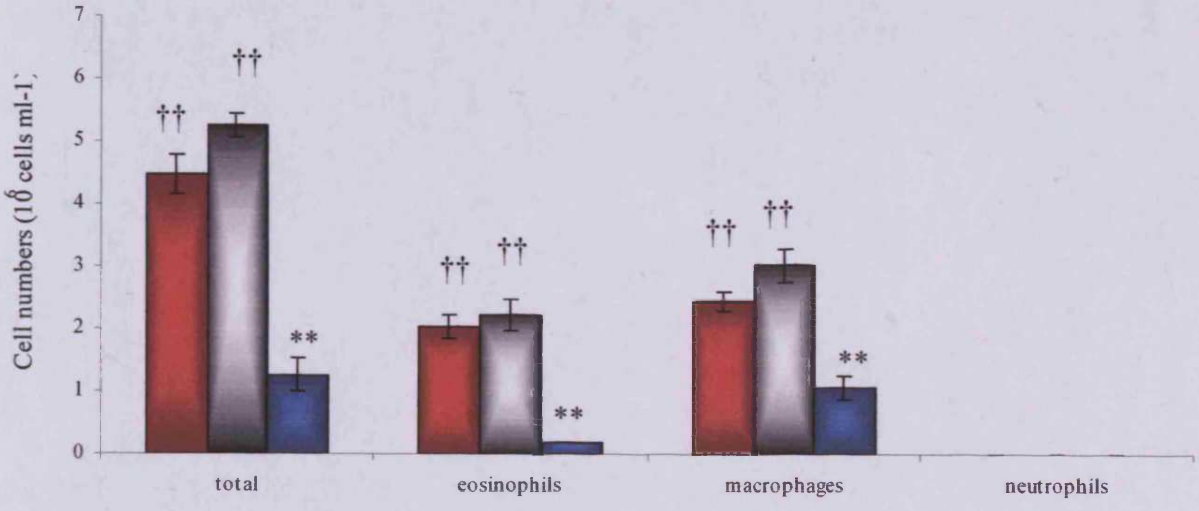


Figure 3.8. The effect of an acute low-dose OA exposure (0.01%/1hr) (■), chronic OA challenge 1 (■) or chronic vehicle challenge (■) on total and differential cell counts in the BALF of sensitised guinea pigs 24hrs subsequent to challenge. Results are expressed as mean ± s.e.m. cell numbers per ml of BALF. n=6. ** ($p<0.01$) significantly different from acute low-dose OA challenge. †† ($p<0.01$) significantly different from chronic vehicle challenge.

3.4.3 CHRONIC OA CHALLENGE 2

In this protocol, the number of OA challenges was increased from 6 OA challenges (chronic OA challenge 1) to 8 OA challenges (chronic OA challenge 2). Sensitised guinea pigs were challenged with a single exposure of nebulised low dose OA (0.01% for 1 hr) on days 15, 17, 19, 21, 23, 25, 27 and 29 (Chronic OA challenge 2). The effect of chronic OA challenge 2 in sensitised guinea pigs on lung function measurements, AHR and BALF total and differential cell numbers was assessed.

3.4.3.1 Effect of chronic OA challenge 2 on lung function in sensitised guinea pigs

Initial low-dose OA challenge of chronic OA challenge 2 (day 15) revealed an EAR, identified as an immediate reduction in sG_{aw} (-63.6 ± 7.5%), which recovered to baseline at 5hrs. This was followed by a LAR, demonstrated as a delayed reduction in lung function (-36.8 ± 12.9 %) between 6 and 8 hours subsequent to OA challenge (Fig. 3.9).

Fig. 3.10 represents the mean-time course for changes in sG_{aw} up to 6hrs subsequent to consecutive low dose OA challenges (chronic OA challenge 2). Attenuation of early phase bronchoconstriction was revealed following repeated low dose OA challenge. On day 29, an immediate reduction in sG_{aw} (-19.16 ± 8.66%) was revealed subsequent to low dose OA challenge.

Guinea pig baseline sG_{aw} values were measured prior to low dose OA challenges on days 15, 17, 19, 21, 23, 25, 27 and 29. Fig. 3.11 represents the % change in baseline sG_{aw} values, compared to day 15 baseline sG_{aw}. A gradual reduction in baseline lung function measurements was revealed in guinea pigs exposed to chronic OA challenge 2. Baseline sG_{aw} values were reduced by 28% between day 15 and day 29.

3.4.3.2 Effect of chronic OA challenge 2 on AHR

Fig. 3.12 represents changes in sG_{aw}, compared to baseline sG_{aw}, following exposure to inhaled nose-only histamine (1mM for 20secs) 24hrs prior to and 24hrs subsequent to chronic OA challenge 2. No AHR was revealed subsequent to the last OA challenge of chronic OA challenge 2.

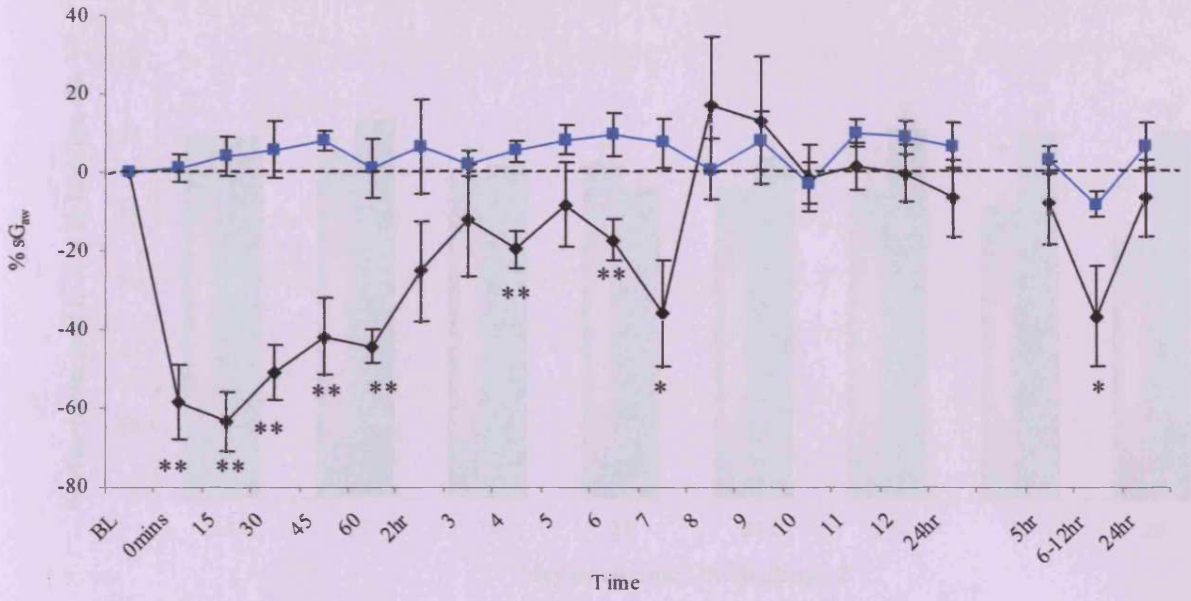


Figure 3.9. The effect of a single low dose OA challenge (day 15 of chronic OA challenge 2) (—◆—) or a single nebulised vehicle challenge (—■—) on lung function measurements in sensitised guinea pigs. Each point represents the mean ± sem change in sG_{aw} compared to baseline sG_{aw}. The mean fall in sG_{aw} between 6 and 12 hours after OA challenge is shown. n=6. ** ($p<0.01$) * ($p<0.05$) significantly different from vehicle.

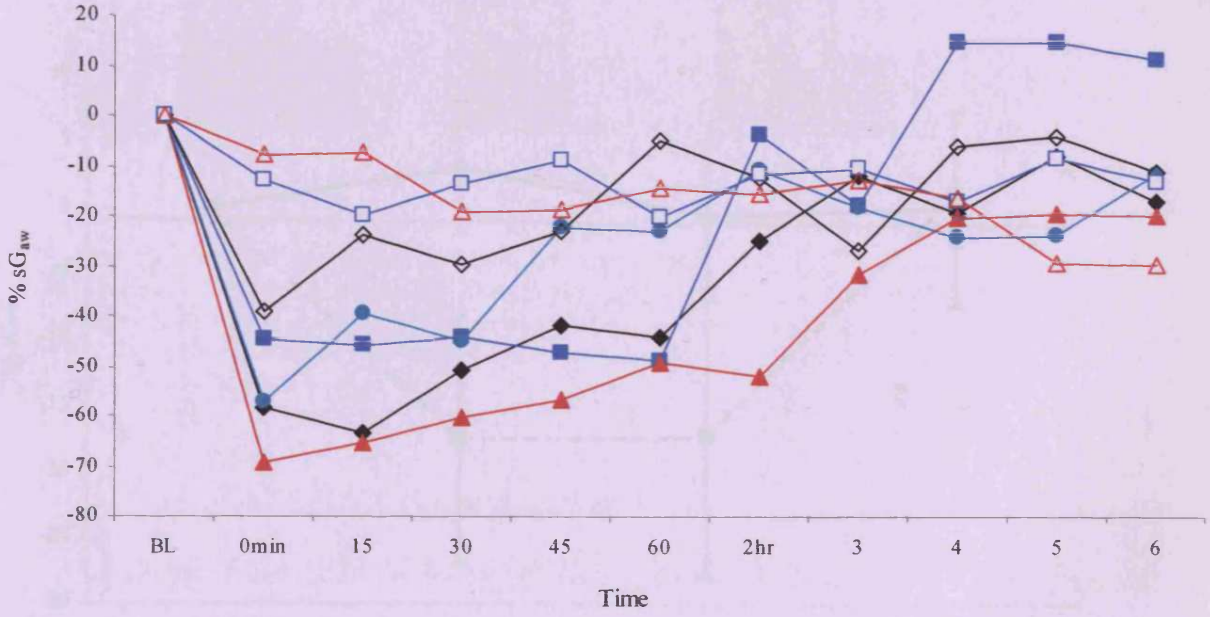


Figure 3.10. The effect of a low dose OA challenge on days 15 (■), 17 (■), 19 (■), 21 (■), 23 (□), no25 (□) and 29(□) on lung function measurements in sensitised guinea pigs, exposed to chronic OA challenge 2. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values up to 6hrs subsequent to consecutive OA challenges.

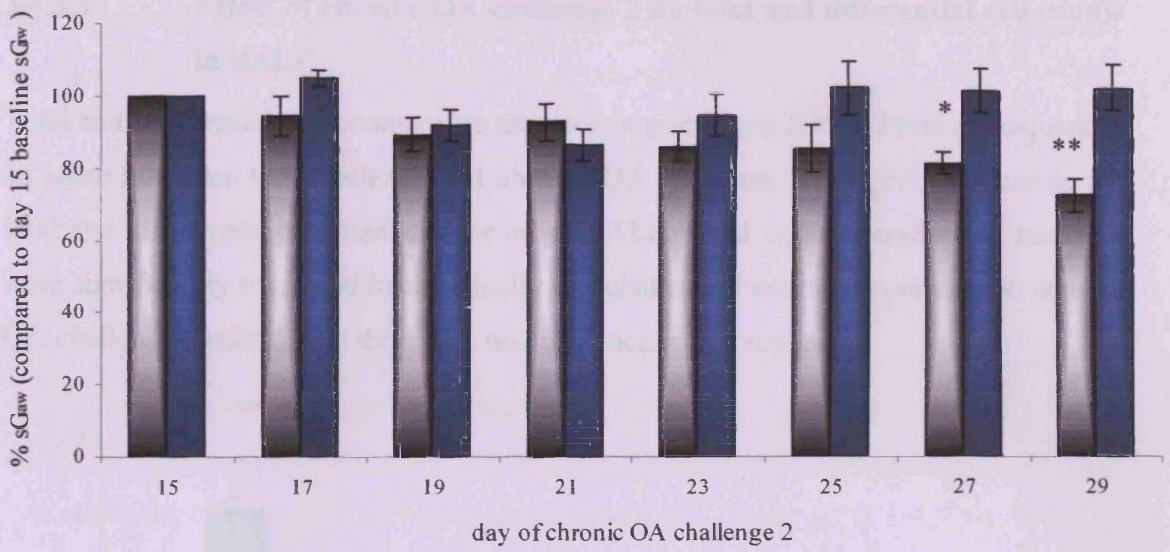


Figure 3.11. The effect of nebulised low dose OA challenges (■) or vehicle challenges (■) on days 15, 17, 19, 21, 23, 25, 27 and 29 on baseline lung function measurements in sensitised guinea pigs. Each point represents the mean ± sem change in baseline sG_{aw} prior to each exposure, compared to day 15 baseline sG_{aw}. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different from vehicle.

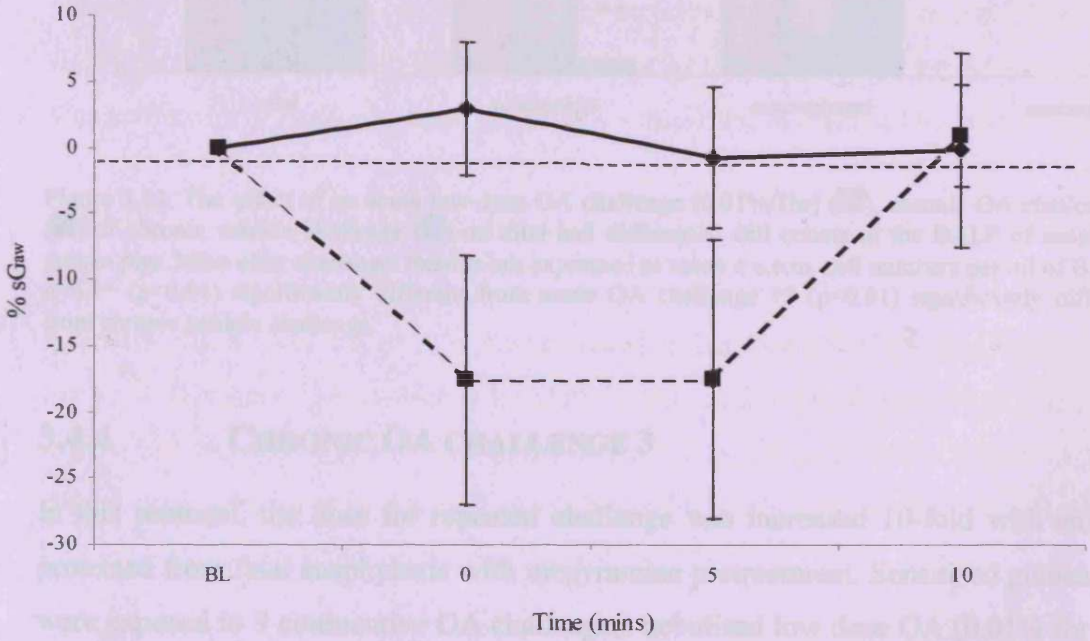


Figure 3.12. The effect of an inhaled nose-only nebulised exposure of histamine (1mM/20secs) 24hrs prior to (◆) and 24hrs subsequent to (■) chronic OA exposure 2 on lung function measurements in sensitised guinea pigs. n=6. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values.

3.4.3.3 Effect of chronic OA challenge 2 on total and differential cell counts in BALF

Total and differential cell counts were assessed in guinea pig BALF 24hrs subsequent to an acute low-dose OA challenge and chronic OA exposure 2. Fig. 3.13 represents the total and differential cell numbers per ml of BALF. Total cell and eosinophil numbers were significantly increased in chronically OA challenged animals, compared to acutely OA challenged animals but there was no difference in macrophages.

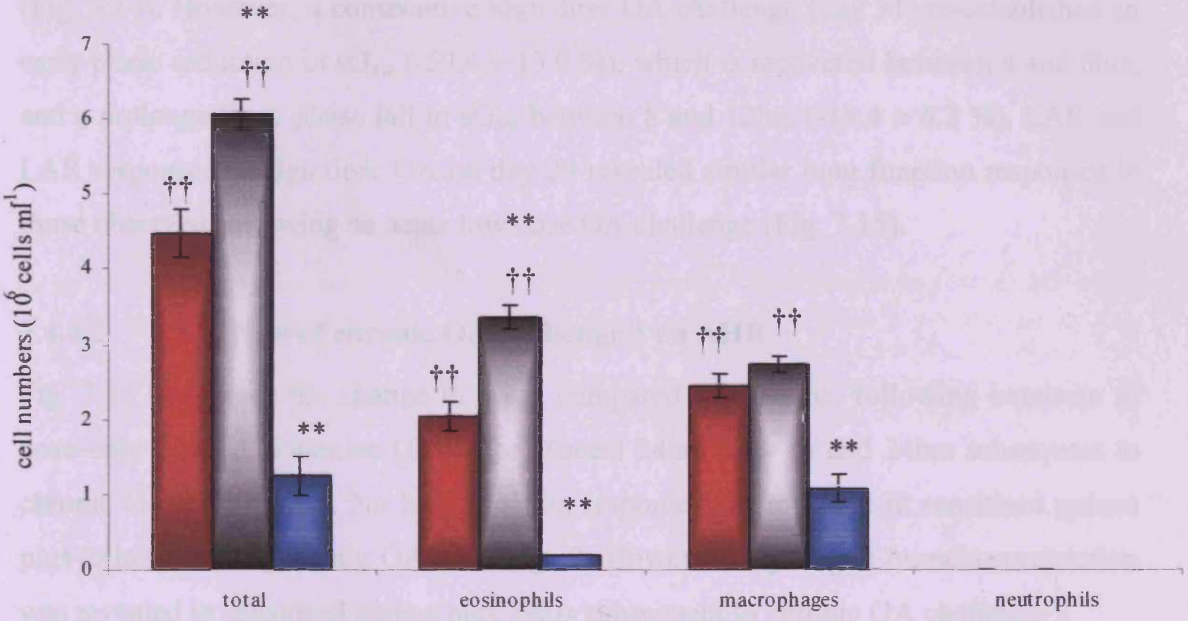


Figure 3.13. The effect of an acute low-dose OA challenge (0.01%/1hr) (■), chronic OA challenge 2 (■) or chronic vehicle challenge (■) on total and differential cell counts in the BALF of sensitised guinea pigs 24hrs after challenge. Results are expressed as mean ± s.e.m. cell numbers per ml of BALF. n=6.** ($p<0.01$) significantly different from acute OA challenge †† ($p<0.01$) significantly different from chronic vehicle challenge.

3.4.4 CHRONIC OA CHALLENGE 3

In this protocol, the dose for repeated challenge was increased 10-fold with animals protected from fatal anaphylaxis with mepyramine pretreatment. Sensitised guinea pigs were exposed to 9 consecutive OA challenges: nebulised low dose OA (0.01% for 1hr) on day 15, mepyramine-protected nebulised high dose OA (0.1% for 1hr) on days 17, 19, 21, 23, 25 and 27, nebulised low dose OA (0.01% for 1hr) on day 29 and finally nebulised high dose OA (0.1% for 1hr) on day 31 (chronic OA challenge 3). The effect

of chronic OA challenge 3 in sensitised guinea pigs on lung function measurements, AHR and BALF total and differential cell numbers was assessed.

3.4.4.1 Effect of chronic OA challenge 3 on lung function in sensitised guinea pigs

Following 6 consecutive mepyramine-protected high-dose OA challenges, a nebulised low-dose OA challenge revealed no EAR or LAR (day 29 of chronic OA challenge 3) (Fig. 3.14). However, a consecutive high dose OA challenge (day 31) re-established an early phase reduction in sG_{aw} (-59.4 ± 13.0 %), which is recovered between 4 and 6hrs, and a prolonged late phase fall in sG_{aw} between 8 and 12hrs (-18.4 ± 6.2 %). EAR and LAR responses to high dose OA on day 29 revealed similar lung function responses to those observed following an acute low dose OA challenge (Fig. 3.15).

3.4.4.2 Effect of chronic OA challenge 3 on AHR

Fig. 3.16 represents the change in sG_{aw}, compared to baseline, following exposure to nose-only inhaled histamine (1mM for 20secs) 24hrs prior to and 24hrs subsequent to chronic OA challenge 3. No lung function response was revealed in sensitised guinea pigs 24hrs prior to chronic OA challenge 3. However, significant bronchoconstriction was revealed in sensitised guinea pigs 24hrs subsequent to chronic OA challenge 3.

3.4.4.3 Effect of chronic OA challenge 3 on total and differential cell counts in BALF

Total and differential cell counts were assessed in guinea pig BALF 24hrs subsequent to an acute low-dose OA challenge or chronic OA challenge. A significant increase in eosinophil numbers was revealed in the BALF of guinea pigs exposed to chronic OA challenge 3, compared to acutely OA challenged animals. However, total cell numbers and macrophage numbers were unchanged (Fig. 3.17).

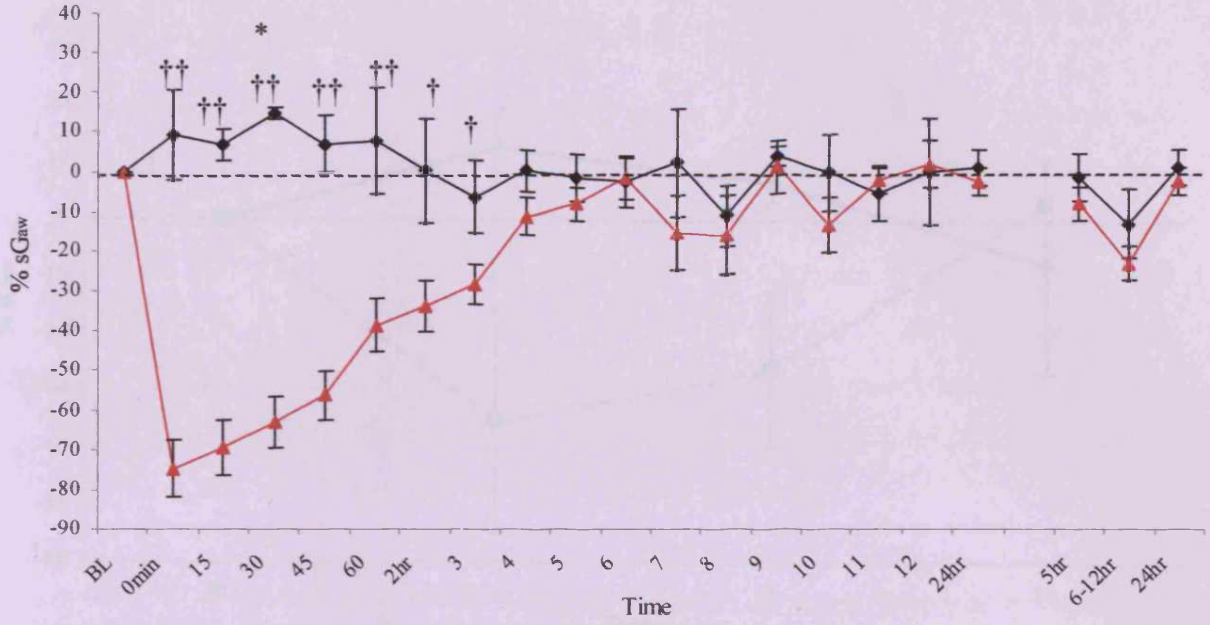


Figure 3.14. Lung function responses following nebulised low-dose OA challenge (day 29 of chronic OA challenge 3) in sensitised guinea pigs (◆). Each point represents the mean ± sem change in sG_{aw} compared to baseline sG_{aw} values. The mean fall in sG_{aw} between 6 and 12hrs after OA challenge is also shown. n=6. The effect of an acute OA challenge (0.01%/1hr) on lung function responses in sensitised guinea pigs is also shown. (▲) n=6. * ($p<0.05$) significantly different from baseline. † ($p<0.05$) †† ($p<0.01$) significantly different from acute OA challenge.

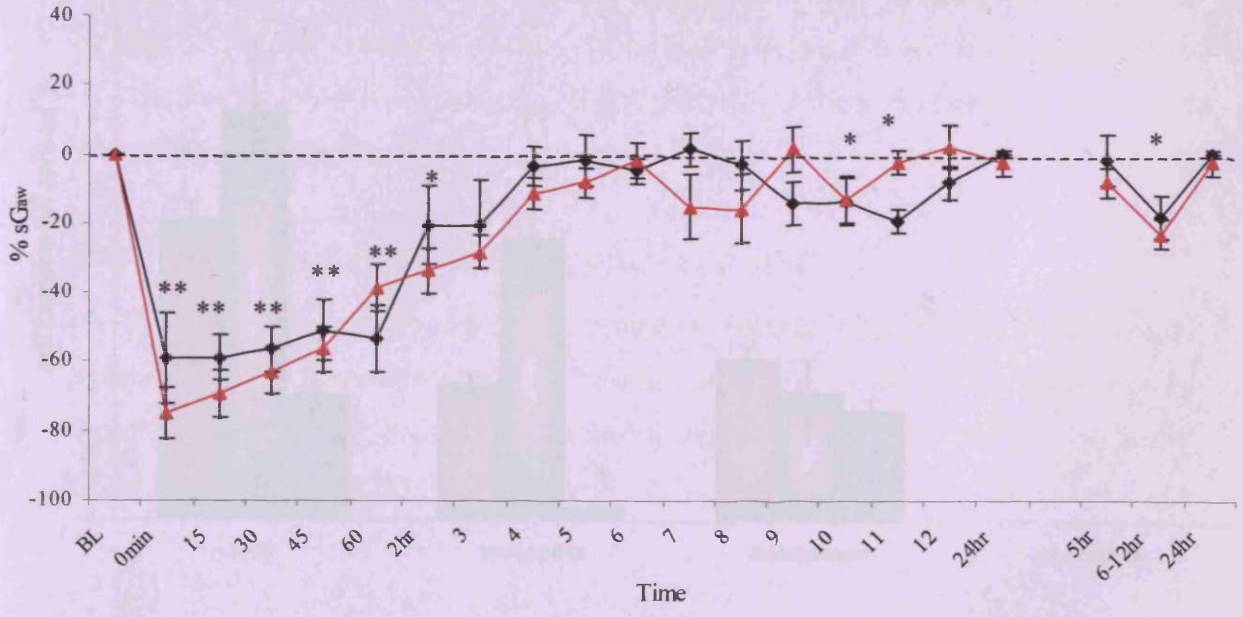


Figure 3.15. Lung function responses following nebulised high-dose OA challenge (day 31 of chronic OA challenge 3) in sensitised guinea pigs (◆). Each point represents the mean ± sem change in sG_{aw} compared to baseline sG_{aw} values. The mean fall in sG_{aw} between 6 and 12hrs after OA challenge is shown. n=6. The effect of an acute OA challenge (0.01%/1hr) (▲) is also shown. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different from baseline sG_{aw}.

3.4.5.3 Effect of chronic OA challenge 4 on total and differential cell counts in BALF

Total cell, eosinophil and macrophage numbers were significantly increased in sensitised guinea pigs exposed to chronic OA challenge 4, compared to guinea pigs exposed chronically to vehicle. Additionally, a significant increase in eosinophil numbers in the BALF of guinea pigs challenged to chronic OA challenge 4, compared to animals exposed to an acute OA challenge was observed (Fig. 3.20).

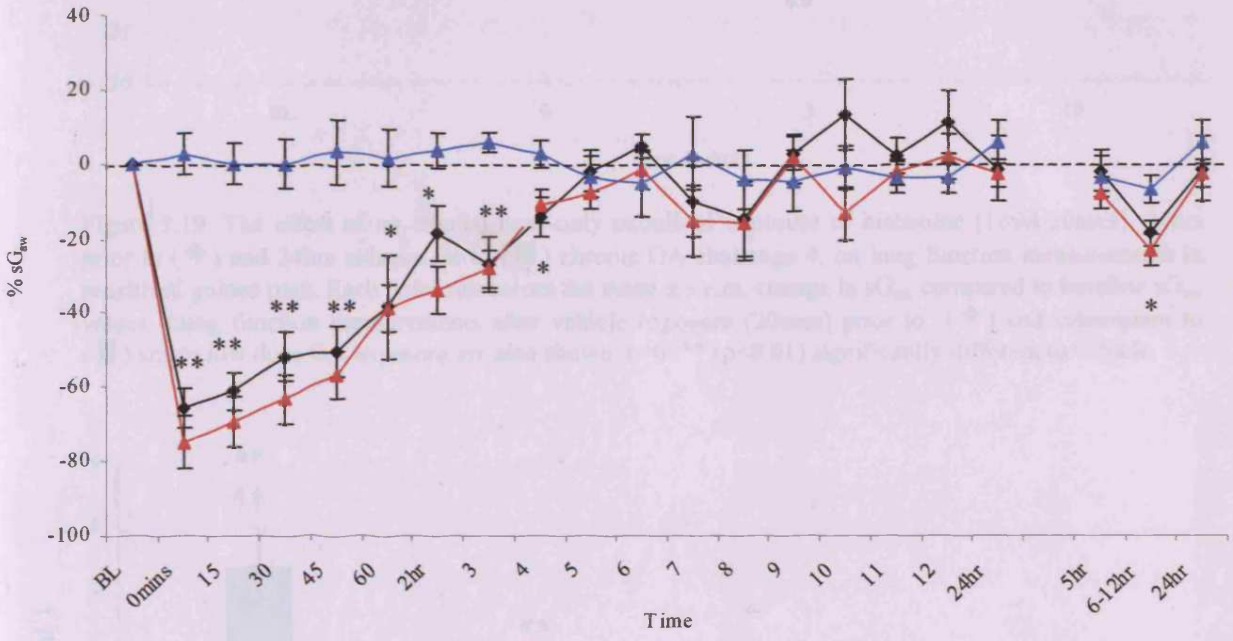


Figure 3.18. Lung function responses following nebulised high-dose OA challenge (0.1%/1hr) (day 29 of chronic OA challenge 4) (◆),compared to an acute OA challenge (0.01%/1hr) (▲) or chronic vehicle exposure (1hr) (▲), in sensitised guinea pigs. Each point represents the mean ± sem change in sG_{aw} compared to baseline sG_{aw} values. The mean fall in sG_{aw} between 6 and 12hrs after OA challenge is shown. (n=6). * ($p<0.05$) ** ($p<0.01$) significantly different from vehicle.

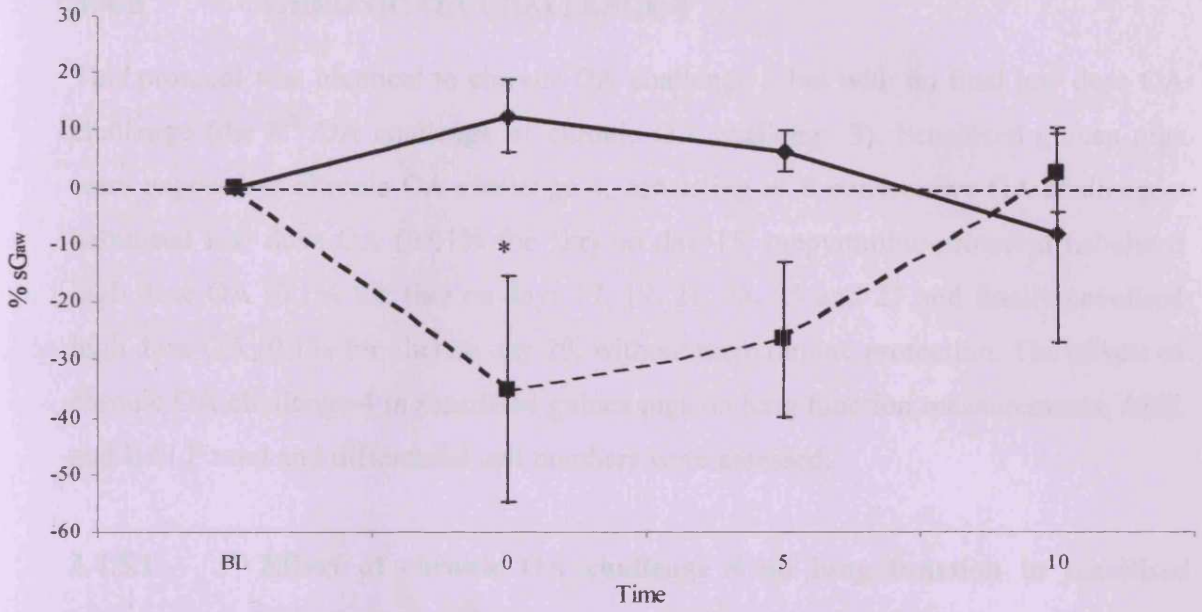


Figure 3.16. The effect of an inhaled nose-only nebulised exposure of histamine (1mM/20secs) 24hrs prior to (◆) and 24hrs subsequent to (■) chronic OA exposure 3 on lung function measurements in sensitised guinea pigs. n=6. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. * significantly different to change in sG_{aw} 24hrs prior to chronic OA challenge 3.

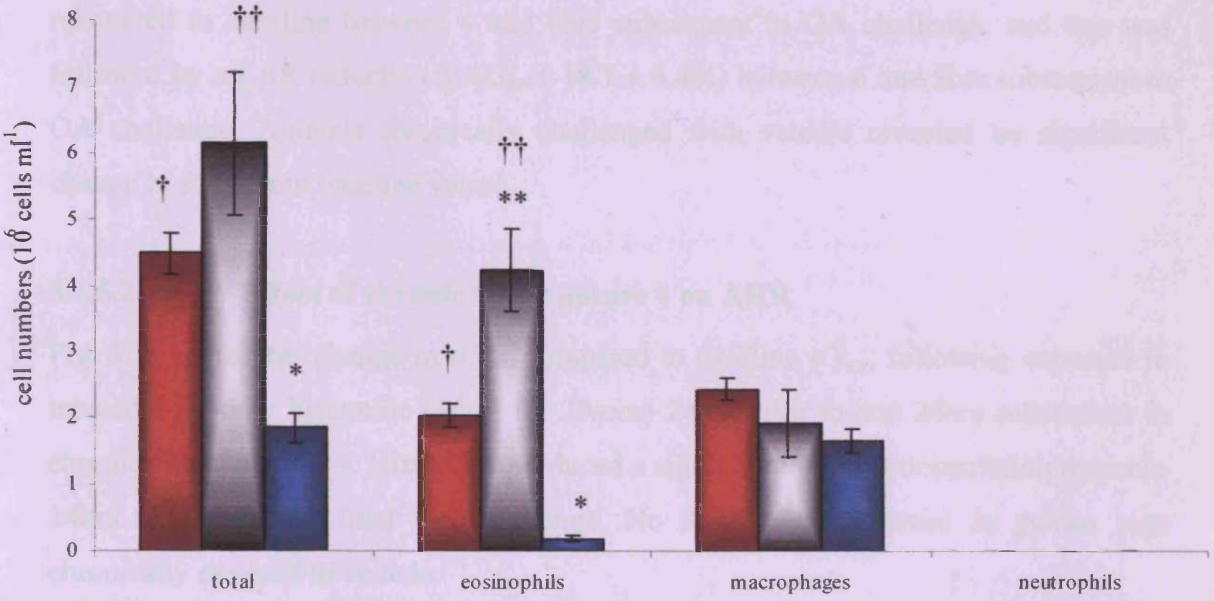


Figure 3.17. The effect of an acute low-dose OA challenge (0.01%/1hr) (▭) or chronic OA challenge 3 (■) on total and differential cell counts in the BALF of sensitised guinea pigs 24hrs after challenge. Results are expressed as mean ± s.e.m. cell numbers per ml of BALF. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different from acute OA challenge. † ($p<0.05$) †† ($p<0.01$) significantly different from chronic vehicle challenge.

3.4.5 CHRONIC OA CHALLENGE 4

This protocol was identical to chronic OA challenge 3 but with no final low dose OA challenge (the 8th OA challenge of chronic OA challenge 3). Sensitised guinea pigs were exposed to chronic OA challenge 4, consisting of 8 consecutive OA challenges: nebulised low dose OA (0.01% for 1hr) on day 15, mepyramine-protected nebulised high dose OA (0.1% for 1hr) on days 17, 19, 21, 23, 25 and 27 and finally nebulised high dose OA (0.1% for 1hr) on day 29, without mepyramine protection. The effects of chronic OA challenge 4 in sensitised guinea pigs on lung function measurements, AHR and BALF total and differential cell numbers were assessed.

3.4.5.1 Effect of chronic OA challenge 4 on lung function in sensitised guinea pigs

Fig. 3.18 represents the mean time course for the changes in sG_{aw} subsequent to the final nebulised high dose OA challenge of chronic OA challenge 4 (day 29) in sensitised guinea pigs. Exposure to nebulised high dose OA on day 29 resulted in an EAR, identified as an immediate reduction in sG_{aw} (-65.6 ± 5.5%). sG_{aw} values were recovered to baseline between 4 and 6hrs subsequent to OA challenge, and this was followed by a LAR reduction in sG_{aw} (-18.7 ± 6.4%) between 6 and 9hrs subsequent to OA challenge. Animals chronically challenged with vehicle revealed no significant change in sG_{aw} from baseline values.

3.4.5.2 Effect of chronic OA exposure 4 on AHR

Fig. 3.19 represents change in sG_{aw}, compared to baseline sG_{aw}, following exposure to inhaled nose-only histamine (1mM for 20secs) 24hrs prior to and 24hrs subsequent to chronic OA challenge 4. Histamine produced a significant bronchoconstriction response 24hrs following the final OA challenge. No AHR was observed in guinea pigs chronically exposed to vehicle.

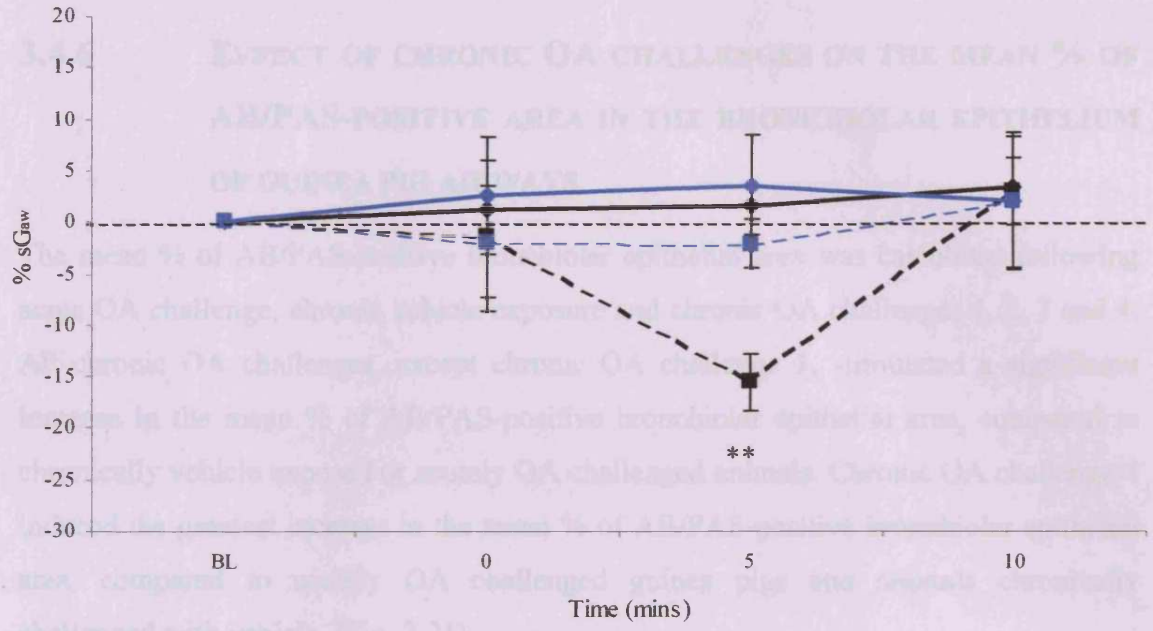


Figure 3.19. The effect of an inhaled nose-only nebulised exposure of histamine (1mM/20secs), 24hrs prior to (◆) and 24hrs subsequent to (■) chronic OA challenge 4, on lung function measurements in sensitised guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. Lung function measurements after vehicle exposure (20secs) prior to (◆) and subsequent to (■) single low dose OA exposure are also shown. n=6. ** ($p<0.01$) significantly different to vehicle.

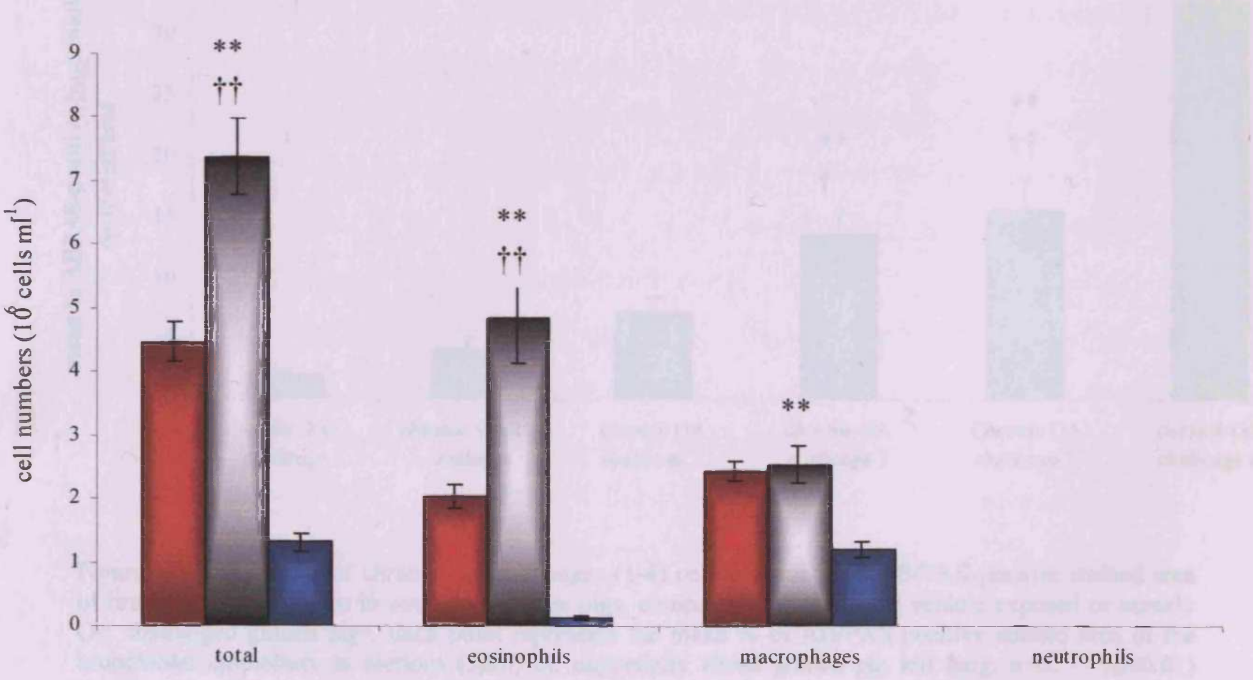


Figure 3.20. The effect of an acute low-dose OA exposure (0.01%/1hr) (■), chronic OA challenge 4 (■) or chronic vehicle exposure (■) on total and differential cell counts in the BALF of sensitised guinea pigs 24hrs after challenge. Results are expressed as mean ± s.e.m. cell numbers per ml of BALF. n=6. †† ($p<0.01$) significantly different to acute OA challenge. ** ($p<0.01$) significantly different to chonic vehicle challenge.

3.4.6 EFFECT OF CHRONIC OA CHALLENGES ON THE MEAN % OF AB/PAS-POSITIVE AREA IN THE BRONCHIOLAR EPITHELIUM OF GUINEA PIG AIRWAYS

The mean % of AB/PAS-positive bronchiolar epithelial area was calculated following acute OA challenge, chronic vehicle exposure and chronic OA challenges 1, 2, 3 and 4. All chronic OA challenges, except chronic OA challenge 1, stimulated a significant increase in the mean % of AB/PAS-positive bronchiolar epithelial area, compared to chronically vehicle exposed or acutely OA challenged animals. Chronic OA challenge 4 induced the greatest increase in the mean % of AB/PAS-positive bronchiolar epithelial area, compared to acutely OA challenged guinea pigs and animals chronically challenged with vehicle (Fig. 3.21).

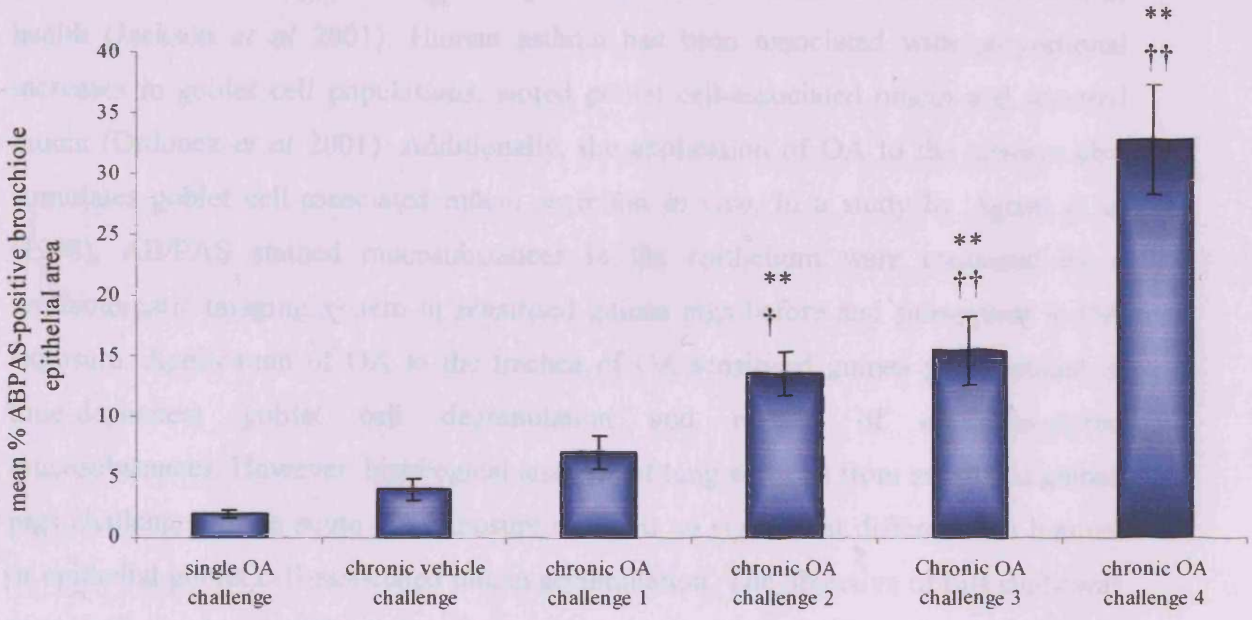


Figure 3.21. The effect of chronic OA challenges (1-4) on the mean % of AB/PAS-positive stained area of bronchiolar epithelium in sensitised guinea pigs, compared to chronically vehicle exposed or acutely OA challenged guinea pigs. Each point represents the mean % of AB/PAS-positive stained area of the bronchiolar epithelium in sections (3μm) of tangentially sliced guinea pig left lung. n=6. ** ($p<0.01$) significantly different to acute OA challenge. n=6. † ($p<0.05$) †† ($p<0.01$) significantly different from chronic vehicle challenge.

3.5 DISCUSSION

3.5.1 ALLERGIC RESPONSES TO INHALED OA IN SENSITISED GUINEA PIGS

Although the acute OA model of asthma has been extensively investigated, there has been significantly less focus on airway responses following chronic allergen challenges. Consistent with findings by Smith and Broadley (2007), the present studies have demonstrated an allergic response following a single nebulised OA challenge in sensitised guinea pigs, characterised by early and late phase bronchoconstriction, AHR, and inflammatory cell recruitment to the airways; all features of human asthma. However, mucus hypersecretion is an additional well-established characteristic of human asthma, which can lead to airway obstruction and contribute to deterioration of health (Jackson *et al* 2001). Human asthma has been associated with proportional increases in goblet cell populations, stored goblet cell-associated mucin and secreted mucin (Ordonez *et al* 2001). Additionally, the application of OA to the airways also stimulates goblet cell-associated mucin secretion *in vivo*. In a study by Agusti *et al* (1998), AB/PAS stained mucosubstances in the epithelium were measured by a semiautomatic imaging system in sensitised guinea pigs before and subsequent to OA exposure. Application of OA to the trachea of OA-sensitised guinea pigs resulted in time-dependent goblet cell degranulation and release of epithelial-stored mucosubstances. However, histological analysis of lung sections from sensitised guinea pigs challenged to an acute OA exposure revealed no significant difference in luminal or epithelial goblet cell-associated mucin accumulation. The objective of this study was to optimise a chronic OA challenge to achieve EAR, LAR, AHR, cellular infiltration and increased goblet cell-associated mucin production in a sensitised guinea pig, which may then be utilised to study the effect of mucus secretagogues in subsequent chapters.

A chronic exposure of 6 consecutive nebulised low dose OA challenges, 48hrs apart, revealed no significant increase in the AB/PAS-positive area of guinea pig bronchiolar epithelium, compared to acutely OA challenged guinea pigs. In addition, chronic low dose OA challenge revealed attenuation of EAR, complete suppression of LAR and a loss of airway hyperreactivity to inhaled histamine, despite persistent inflammatory cell

recruitment to the airways. In order to reveal the pattern of tolerance to chronic OA challenge, sensitised guinea pigs were challenged with 8 consecutive low dose OA exposures, 48hrs apart (chronic OA challenge 2) and a time course study of sG_{aw} measured up to 6hrs subsequent to each exposure. Attenuation of immediate early phase bronchoconstriction was revealed following consecutive low dose OA challenges, demonstrating that repeated low dose allergen exposures can induce gradual alleviation of EAR.

Initiation of the EAR is dependent on the degranulation of mast cells subsequent to mast cell-fixed IgE and allergen cross-linking following OA exposure. Resulting mast cell degranulation leads to the subsequent release of numerous mediators including histamine, chymase, tryptase and interleukins (Hart 2001). Histamine was one of the first mediators implicated in asthma and stimulates EAR bronchoconstriction following its release via the activation of H_1 receptors. The LAR is believed to be a consequence of the effects of activated inflammatory cells, which are recruited to the lungs by mast cell-derived mediators (Hart 2001). Therefore, attenuation of EAR and loss of LAR following chronic low dose OA challenge may have been a result of potential depletion of the activated OA-specific mast cell population due to repeated OA-induced mast cell degranulation. However, an adequate mast cell population and inflammatory cell influx was confirmed in guinea pigs previously challenged with chronic low dose OA and unresponsive to low dose OA. In these animals, a high dose OA exposure revealed extensive EAR bronchoconstriction and potential anaphylaxis (lung function results not available), a response that is induced by mast cell derived histamine.

Chronic exposure to nebulised low dose allergen has previously revealed tolerance in a dose dependent manner (Schramm *et al* 2004). The third exposure protocol (chronic OA challenge 3) utilised high-dose OA exposures to potentially reinstate responsiveness to nebulised OA. However, acutely low dose OA challenged guinea pigs could not tolerate a nebulised exposure of high dose OA and therefore high dose OA exposures were preceded with an i.p. injection of mepyramine (30mg/kg) to protect against fatal anaphylaxis. A single low-dose OA exposure failed to stimulate an EAR or LAR in sensitised guinea pigs previously challenged with mepyramine-protected chronic high-dose OA. However, subsequent to mepyramine-protected chronic high-dose OA challenge, guinea pigs airways became conditioned to withstand nebulised high dose

OA exposure without mepyramine protection. Furthermore, high dose OA challenge resulted in the development of an allergic airway response to an extent similar to that observed in the acute model, with a shift in the LAR from between 6 and 10hrs to between 9 and 12hrs post OA exposure.

The established model of chronic asthma (chronic OA challenge 4) consisted of one low dose OA exposure and seven consecutive high dose OA exposures, all but the ultimate exposure preceded by an i.p. injection of mepyramine to protect against fatal anaphylaxis. The ultimate high dose OA exposure revealed all human asthma responses demonstrated following acute challenge including EAR, LAR, AHR and inflammatory cell infiltration into the airways. Additionally, histological analysis revealed significant goblet cell-associated mucin accumulation compared to guinea pigs exposed to a single OA challenge and control animals (17.1 fold and 8.3 fold increase respectively).

3.5.2 TOLERANCE IN SENSITISED AND CHRONICALLY OA CHALLENGED GUINEA PIGS

Allergen exposure in an atopic individual stimulates a Th2 immune response, characterised by the development of allergen-specific IgE and IgG1 antibodies and production of inflammatory cytokines such as IL-5, IL-3, IL-6, IL-13 and IL-4, which evoke eosinophilic recruitment, goblet cell differentiation and mucin production (Wills-Karp 1999). In the sensitised guinea pig, an acute OA exposure stimulates a classic Th2 immune response (Smith and Broadley 2007). The Th2 response is characterised by increased circulating IgE. However, it is widely accepted that moderation of allergy can be achieved through repeated allergen exposure (Platts-Mills *et al* 2003). The phenomenon of tolerance, developed in response to repeated allergen exposures, was observed following chronic OA challenge in our guinea pig model of asthma. These results are consistent with findings by Schramm *et al* (2004), which revealed unresponsiveness to OA on repeated exposure in a mouse model of asthma. However, although research into the induction of tolerance has been considerable due to the potential use of induced tolerance as a treatment for allergic disease, the exact mechanisms behind the development of tolerance are unclear. Several proposed mechanisms of immunological respiratory tolerance include clonal deletion, anergy, immune deviation and active suppression, as described in the introduction to this

chapter. A description and discussion of the possible role of these mechanisms in the guinea pig model of chronic asthma is presented below.

Clonal deletion involves activation-induced apoptosis and subsequent elimination of memory T cells, resulting in a loss of responsiveness to an allergen that has previously induced an allergic response, and prevention of a further allergic response to allergen challenge. Anergy is characterised by functional inactivation of peptide specific CD4+ T cells. It requires continuous interaction between allergen and the TCR and therefore is maintained by the persistence of peptide. If tolerance to inhaled OA had been induced via clonal deletion or anergy in the guinea pig model of chronic asthma, tolerance should have been sustained despite allergen dose. However, exposure to nebulised high dose OA (10 fold increased) subsequent to chronic low dose OA resulted in the development of allergic airway disease to an extent similar to that observed in animals acutely exposed to low dose OA.

Regulation or suppression of an immune response, via the suppression of Th2 cell growth and proliferation can be mediated by the production of antigen-specific regulatory T cells (T_{reg}), via the secretion of regulatory cytokines such as IL-10, IL-4 and TGF-β. Additionally, γδ CD8+ T cells can play a role in maintaining tolerance to allergic response by inducing immune deviation from a Th2 immune response to a Th1 immune response via the secretion of inhibitory cytokines such as IFN-γ (McMenamin and Holt 1993). However, inhibition of IgE synthesis by γδ CD8+ T cell-derived IFN-γ or inhibition of Th2 cell proliferation by T_{reg} cells should be accompanied by alleviation of all Th2 responses. However, a split model of tolerance was observed in the guinea pig model of chronic low-dose OA challenge, characterised by the alleviation of some allergic responses (EAR, LAR and AHR), but not others (eosinophilia or goblet cell-associated mucin accumulation).

3.5.3 EOSINOPHILIA IN TOLERANT GUINEA PIGS

As previously described, the guinea pig model of chronic low-dose OA challenge was characterised by alleviation of EAR, LAR and AHR despite persistence or an increase in eosinophilia (chronic OA challenge 1 and chronic OA challenge 2 respectively). Eosinophilia is considered an important factor in allergic airway disease and the relative number of recruited eosinophils in the lungs corresponds to the severity of asthma.

Eosinophils respond to a number of chemoattractants including complement factor C5a, PAF, LTB4, IL-2, IL-3, IL-5, IL-16, eotaxin, IL-8 and RANTES (Resnick and Weller 1993). IL-5 and eotaxin are produced during a Th2 response and maintain eosinophil recruitment to the lungs during an allergic response (Hogan *et al* 1998).

The activity of eosinophil peroxidase (EPO) is commonly employed as a measure of eosinophil activation. However, this method was not utilised in the present study due to time restraints. In OA sensitised and chronically challenged guinea pigs, continued eosinophilia in the absence of AHR, EAR and LAR may be due to the persistence of eosinophils in the airways subsequent to OA challenge in the absence of eosinophil activation.

Additionally, T cell activation may be a hierarchical process in which different effector functions are induced at different densities of allergen (Korb *et al* 1999). For example IFN-γ production requires a lower concentration of MHC-peptide than is required for IL-2 production (Korb *et al* 1999), suggesting that activation of specific T cell functions without other responses may be achieved by low dose allergen exposure. It is possible therefore that in the guinea pig model of chronic asthma, consecutive low-dose OA induces the production of IFN- γ, which sequesters eosinophils to the airways, without evoking IgE production. Measurement of IgE in chronically OA challenged guinea pigs would establish whether this is the case.

It has also been previously proposed that γδ CD8+ T cells may promote tolerance to inhaled antigen and inhibit AHR, independent of induction of eosinophilia (Lahn *et al* 1999). Research by Svensson *et al* (2003) demonstrated that TCR γδ knockout mice revealed diminished eosinophilia in OA sensitised and challenged mice, indicating that γδ CD8+ T cells may contribute to eosinophilia. Additionally, studies have shown that eosinophilia can occur in IgE deficient mice (Melhop *et al* 1997). Promotion of eosinophil airway recruitment may also be stimulated by Th1 or γδ CD8+ T cell-derived cytokines. IFN-γ, the inhibitory cytokine secreted by γδ CD8+ T cells, did not inhibit eotaxin production in mice airways and may stimulate eotaxin expression in lung epithelial cells in a dose dependent manner (Li *et al* 1998, Lilly *et al* 1997). In addition to being released during the Th2 response, eotaxin, the potent eosinophil chemoattractant, is secreted from both lung fibroblasts and in human airway smooth muscle cells by the Th1 cytokine, TNFα (Sato *et al* 2001, Pang and Knox 2001),

indicating Th1-mediated eosinophil recruitment. It can therefore be proposed that in our model, immune deviation from a Th2 to a Th1 response mediated by the γδ CD8+ T cells may demonstrate split suppression of Th2 responses resulting in continued eosinophilia, despite attenuation of EAR, LAR and AHR.

Finally, respiratory tolerance may involve multiple and overlapping mechanisms. Active suppression, clonal deletion and anergy have previously been linked (Akbari *et al* 2001). It is likely that the development of tolerance in allergic models is a result of overlapping and linked mechanisms mediated by several cell types and secreted cytokines, rather than due to one distinct pathway. Tolerance to inhaled OA may be induced by alternate mechanisms in different species, and may be dependent on allergen dose, the length of allergen exposure and time intervals between exposures.

Further investigations, for example utilisation of Enzyme Linked Immunosorbent Assays (ELISAs) to further investigate airway BALF cytokine profiles, could provide additional information regarding the mechanisms of tolerance involved in this model. However, although this may provide interesting and valuable information regarding the mechanism of tolerance to allergen in the sensitised guinea pig, it would remove focus from the initial objective of this study.

3.5.4 INFLAMMATORY CELL RECRUITMENT AND AHR IN CHRONICALLY OA CHALLENGED GUINEA PIGS

An increase in inflammatory cell recruitment to the airways was observed in sensitised guinea pigs chronically challenged with both low-dose and high-dose OA, compared to acutely challenged animals. However, although increased eosinophil and macrophage numbers were revealed, no neutrophilic infiltration was detected. Studies by Agusti *et al* (1998) revealed that OA stimulates early selective recruitment of neutrophils 1hr following instillation and that the EAR is neutrophilic. As neutrophils are the first inflammatory cells sequestered to the lungs during an inflammatory response, the removal of airway neutrophils before BAL is likely. A significant increase in the number of eosinophils in BALF was observed following chronic challenge compared to an acute challenge. Eosinophil recruitment to the airways occurs at 8 to 11hrs and is maximal at 24hrs subsequent to allergen challenge (Agusti *et al* 1998). Eosinophil airway infiltration is an important characteristic of human asthma and allows

differentiation between asthma and other airway inflammatory diseases (Barnes and Drazon 2002). Eosinophilia has been associated with AHR for a considerable time and there is evidence both linking AHR and eosinophila (Brusasco *et al* 1990) and disputing the association of AHR and eosinophilia (Birrell *et al* 2003). The present findings revealed that increased BALF eosinophil cell counts occurred independent of AHR. The activation state of the inflammatory cells present in BALF however was not investigated and it is possible that not all recruited inflammatory cells were activated.
Studies by Wu *et al* (2001) suggest that in sensitised and challenged mice, eosinophilia can occur independent of excess mucus production. This was also observed in our model. In guinea pigs, chronically challenged to low-dose OA (chronic OA challenge 1), increased eosinophilia was not associated with increased mucus production. However, in chronically high-dose OA challenged guinea pigs (chronic OA challenge 4) a further increase in eosinophilia, compared to chronically low-dose OA challenged guinea pigs, was associated with significantly increased goblet cell associated mucin accumulation.

3.5.5 MUCUS ACCUMULATION

Accumulation of epithelial stored mucin appears to be dependent on a number of factors. Epithelial stored mucin, identified as the mean % of AB/PAS-positive bronchiolar epithelial area in histological paraffin sections of lung, was measured in the airways of guinea pigs challenged with chronic OA challenges of different doses and number of exposures. Goblet cell-associated mucin accumulation did not appear to be proportional to the number of OA exposures. Levels of epithelial stored mucin were increased in animals exposed to 6 consecutive low-dose OA challenges (3.7 fold and 1.8 fold compared to a single OA challenge and chronic vehicle challenge respectively). However, goblet cell associated mucin accumulation increased dramatically following 2 further low dose OA challenges (7 fold and 3.4 fold compared to a single OA exposure or chronic vehicle exposure respectively).
Additionally, interruption of continuous high-dose OA exposure appeared to have a significant effect on epithelial mucin accumulation. The mean % of AB/PAS bronchiolar epithelial area was significantly greater in animals exposed to continuous high dose OA challenges (chronic OA challenge 4), compared to animals which

received high dose OA challenges interrupted with a single low dose OA challenge (chronic OA challenge 3). Furthermore, increases in airway mucin accumulation following chronic low dose challenges were observed despite reduction in the severity of EAR, loss of LAR and AHR, indicating that EAR, LAR or AHR are not requirements for goblet cell-associated mucin accumulation within the airways.

In conclusion, repeated exposure to high dose OA challenges provides a chronic model of asthma, characterised by EAR, LAR, AHR, inflammatory cell recruitment to the airways and goblet cell-associated mucin accumulation. This model will be utilised in subsequent chapters to evaluate the effects of mucus secretagogues on guinea pig airway responses.

CHAPTER 4

The effects of nucleotide exposure on goblet cell-associated mucin secretion and lung function changes in chronically OA challenged guinea pigs.

4.2 INTRODUCTION

4.2.1 EXTRACELLULAR NUCLEOTIDES

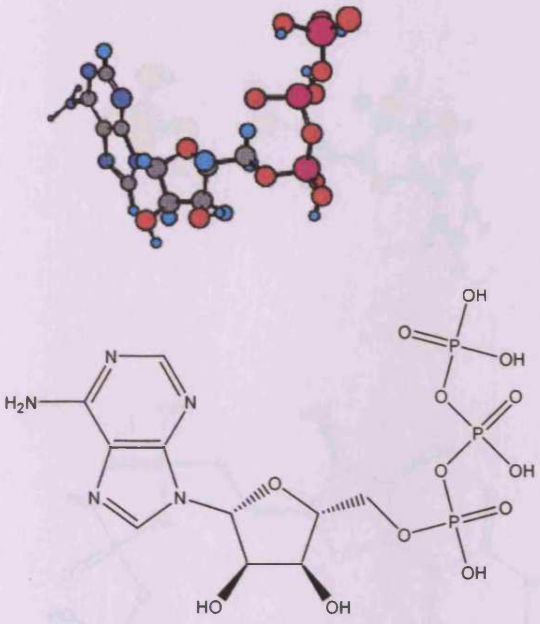
In addition to having a vital role in energy metabolism, the nucleotide adenosine triphosphate (ATP) stimulates a myriad of effects throughout the body. The role of ATP as a neurotransmitter, being co-released with noradrenaline and acetylcholine from sympathetic and parasympathetic nerves respectively, was proposed by Burnstock in the 1970s and is now firmly established (Burnstock 1972). More recently, ATP has been implicated as an extracellular signalling molecule, exerting a wide range and increasing number of physiological effects via a large number of purinergic receptors including smooth muscle contraction, platelet aggregation and inflammation (Banks *et al* 2006, Ts'ao *et al* 1976).

Synthesis of ATP occurs in the mitochondria via oxidative phosphorylation. Subsequent to its synthesis, ATP is transported to the cytosol and stored in cytosolic vesicles at concentrations of 3-5mM in the presence of accompanying biogenic amines. The principal mechanism of ATP cellular release is believed to be via the exocytotic fusion of cytoplasmic vesicle and plasma membrane. However, in addition to exocytosis, additional suggested mechanisms for ATP cellular release include the concentration gradient-dependent release through ATP-permeable channels, transport via P-glycoprotein (P-gp) or transport via the cystic fibrosis transmembrane transporter (CFTR) (Abraham *et al* 1993, Rostovtseva and Bezrukov 1998). Once released from the cell, ATP is free to bind to purinergic (P) receptors or may be degraded by a family of ectoenzymes into the nucleotides ADP and AMP and the nucleoside adenosine, which also have pharmacological significance. For the chemical structures of ATP, ADP, AMP and adenosine refer to Fig. 4.1a-d.

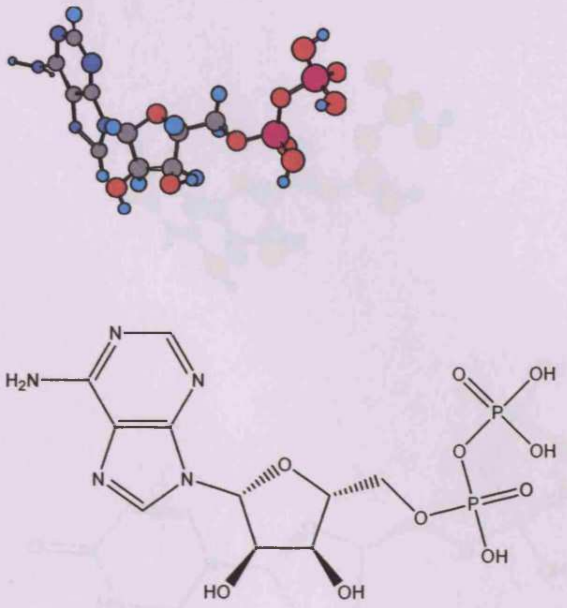
Uridine triphosphate (UTP) demonstrates a non-uniform distribution throughout the body with diverse concentrations in different cells and tissues (Traut 1994). UTP is stored in chromaffin cells, mast cells and platelets at a concentration approximately 10% of ATP levels (Anderson and Parkinson 1997) and intracellular levels are maintained by two biologically important pathways; the de novo biosynthesis pathway and the salvage pathway. These pathways have been reviewed by Anderson and Parkinson (1997). De novo biosynthesis of UTP predominates mainly during cell

growth but is also exploited in the liver (Traut and Jones 1996). It involves the enzymes dihydroorotate synthetase, UMP synthetase and the inner mitochondrial dihydroorotate dehydrogenase. Alternatively, the salvage pathway involves the utilisation and recycling of extracellular uridine. Subsequent to cellular uptake, uridine is phosphorylated into UMP, UDP and UTP by monophosphate kinases. The salvage pathway maintains intracellular uridine nucleotide levels in all except hepatic tissues (Traut and Jones 1996). For the chemical structures of UTP, UDP, UMP and uracil, refer to Fig. 4.1e-h.

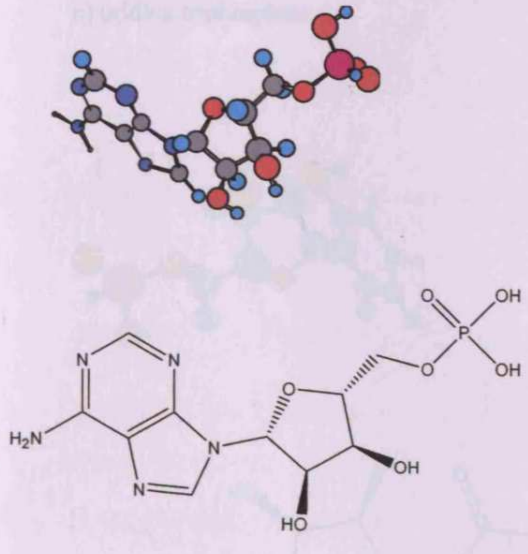
Unlike ATP, which is well established as a neurotransmitter, there is little evidence of synaptic storage of UTP or regulated release of UTP at nerve terminals. However, cellular release of UTP has been demonstrated in several cell types. Basal UTP cellular secretion was firstly revealed in vascular endothelial cells by Saiag *et al* (1995) and this was soon followed by the demonstration of stimulated UTP release in airway epithelial cells, leukocytes and platelets (Lazarowski and Harden 1999). Most recently, an increase in plasma UTP was identified in patients with coronary heart disease, providing evidence of stimulated UTP release in humans (Wihlborg *et al* 2006). The identification of UTP cellular release, along with the discovery of P2Y receptors specific for uridine nucleotides over adenosine nucleotides, encouraged the theory that UTP may act as an extracellular signalling messenger. The activation of UTP-sensitive purinergic receptors has been shown to exert diverse effects on a variety of cells and tissues throughout the body, including muscle cell proliferation (Michoud *et al* 2002), endothelial cell adhesion (Seye *et al* 2003) and mucociliary clearance (MCC) (Olivier *et al* 1996).



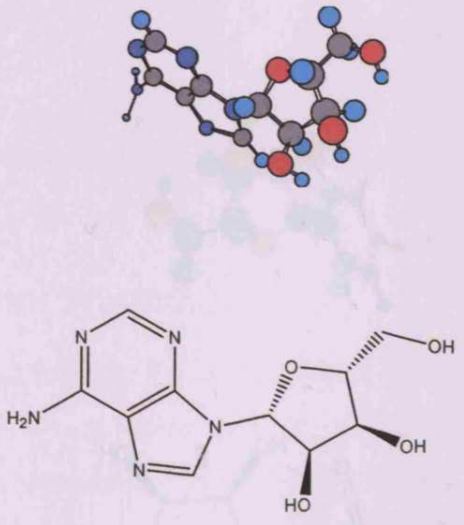
a) adenosine triphosphate



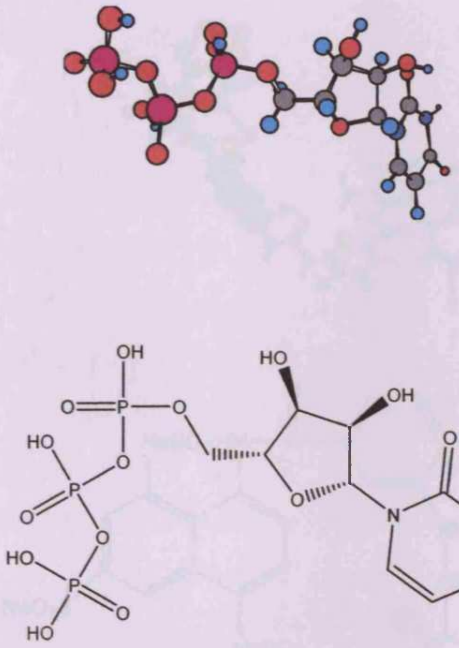
b) adenosine diphosphate



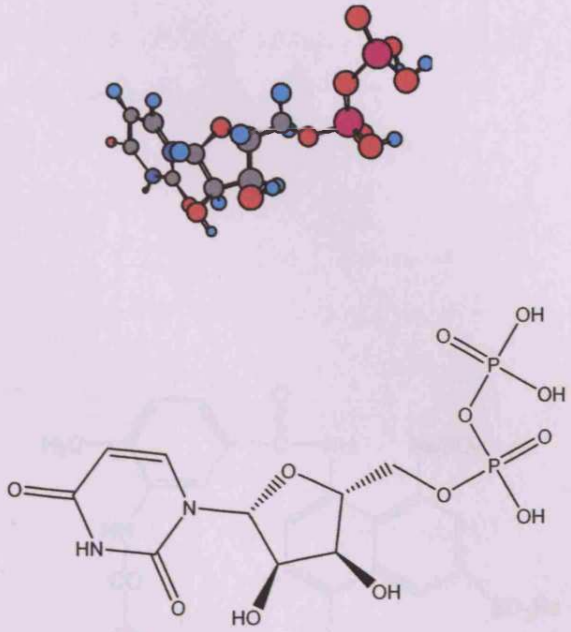
c) adenosine monophosphate



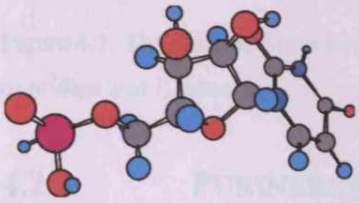
d) adenosine



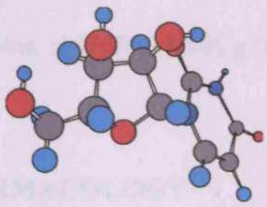
e) uridine triphosphate



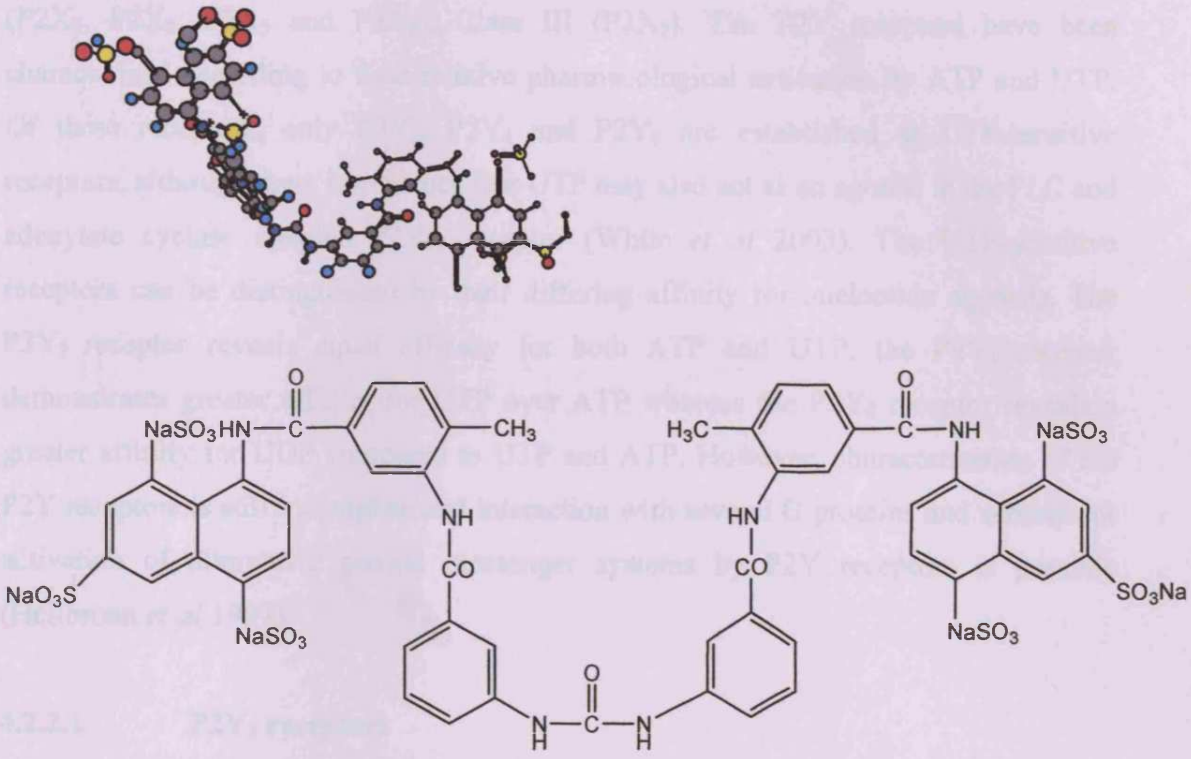
f) uridine diphosphate



g) uridine monophosphate



h) uridine



i) suramin

Figure 4.1. The chemical structures of a) ATP, b) ADP, c) AMP, d) adenosine, e) UTP, f) UDP, g) UMP, h) uridine and i) suramin

4.2.2 PURINERGIC AND PYRIMIDINERGIC PHARMACOLOGY

Purinergic and pyrimidinergic receptors are divided into the P1 receptors and the P2 receptors according to their relative affinities for purines and their signalling pathways. Four distinct subtypes of P1 receptors have been characterised including the A_1, A_{2a}, A_{2b} and A_3; all of which are activated by adenosine and coupled to either adenylate cyclase or phospholipase C. The P2 receptors have been extensively studied and well characterised. They are activated by ATP and UTP and can further be divided into the ligand-gated ionotropic P2X receptors and the G-protein-coupled P2Y receptors, of which the signalling cascades are illustrated in Table 4.1 (adapted from Burnstock and Williams (2000) and Brunschweiger and Muller (2006)). Seven P2X receptors ($P2X_{1-7}$) and eight P2Y receptors have been characterised ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$). P2X receptors can be grouped into 3 classes according to

their affinity for ATP and level of desensitisation: Class I ($P2X_1$ and $P2X_3$), Class II ($P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$), Class III ($P2X_7$). The P2Y receptors have been characterised according to their relative pharmacological activation by ATP and UTP. Of these receptors, only $P2Y_2$, $P2Y_4$ and $P2Y_6$ are established as UTP-sensitive receptors, although there is evidence that UTP may also act as an agonist at the PLC and adenylate cyclase coupled $P2Y_{11}$ receptor (White *et al* 2003). The UTP-sensitive receptors can be distinguished by their differing affinity for nucleotide agonists. The $P2Y_2$ receptor reveals equal efficacy for both ATP and UTP, the $P2Y_4$ receptor demonstrates greater affinity for UTP over ATP whereas the $P2Y_6$ receptor reveals a greater affinity for UDP compared to UTP and ATP. However, characterisation of the P2Y receptors is still incomplete and interaction with several G proteins and subsequent activation of alternative second messenger systems by P2Y receptors is possible (Heilbronn *et al* 1997).

4.2.2.1 $P2Y_2$ receptors

The $P2Y_2$ receptor is the only P2 receptor that is equally stimulated by the structurally different compounds, ATP and UTP. The affinity of UDP at $P2Y_2$ receptors however, is questionable. There is contrasting evidence that UDP is both inactive (Nicholas *et al* 1996) and a partial agonist at $P2Y_2$ receptors (Shen *et al* 2004). $P2Y_2$ receptors are widely distributed throughout the body and are found in all the body's mucosal surfaces including lungs, gastrointestinal tract, vaginal tract, eyes and mouth (Inspire Pharmaceutics 2007). In the airways $P2Y_2$ receptors are found on the surface of goblet cells, ciliated epithelial cells, Type II alveolar cells and cells of the immune system (T cells and monocytes) (Inspire Pharmaceutics 2007). The $P2Y_2$ receptors are G-protein coupled receptors, which stimulate phospholipase C and inositol triphosphate to initiate mobilisation of intracellular Ca^{2+} and activation of PKC.

Receptor subtype	Signal transduction	Agonists	Antagonists
$P2X_1$	$I_{Na/K/Ca^{2+}}$	ATP 2-MeSATP α-β-MeATP	IsoPPADS MRS2159 Suramin Phenol red TNP-ATP TNP-GTP PPADS
$P2X_2$	$I_{Na/K}$	ATP 2-MeSATP	PPADS Suramin TNP-ATP Reactive blue 2
$P2X_3$	$I_{Na/K/Ca^{2+}}$	ATP 2-MeSATP α-β-MeATP	TNP-ATP TNP-GTP Suramin Phenol red IsoPPADS NF023
$P2X_4$	$I_{Na/K}$	ATP 2-MeSATP CTP	Brilliant blue G TNP-ATP Phenolphthalein
$P2X_5$	$I_{Na/K/Ca^{2+}}$	ATP > ADP 2-MeSATP α-β-MeATP BzATP GTP	Brilliant blue G PPADS Suramin TNP-ATP
$P2X_6$	$I_{Na/K/Ca^{2+}}$	ATP > ADP α-β-MeATP	isoPPADS TNP-ATP
$P2X_7$	$I_{Na/K}$	ATP > ADP 2-MeSATP BzATP	Brilliant blue G
$P2Y_1$	$G_{q/11}$ (increase IP_3/DAG)	ATP = ADP 2-MeSATP 2-MeSADP MRS2365	Suramin PPADS Reactive blue-2 MRS2179 MRS2500 MRS2279 BzATP
$P2Y_2$	$G_{q/11}$ (increase IP_3/DAG)	UTP = ATP UDP? INS37217 INS365	Suramin Reactive blue 2
$P2Y_4$	$G_{q/11}$ (increase IP_3/DAG)	UTP = UDP ATP = ADP	Reactive blue 2 PPADS

		INS37217 INS365	BzATP
$P2Y_6$	$G_{q/11}$ (increase IP_3/DAG)	UDP > UTP > ADP > ATP	MRS2578 Reactive blue 2 PPADS Suramin
$P2Y_{11}$	$G_{q/11}$ (increase IP_3/DAG) or Gs (increase in cAMP)	ATP > ADP (possibly UTP)	Suramin Reactive blue 2
$P2Y_{12}$	G_i (cAMP modulation)	ADP 2-MeSADP IDP	ATP AR-C69931MX ARC67085MX 2-MeSAMP Reactive blue 2 Suramin BzATP
$P2Y_{13}$	G_i (cAMP modulation)	ADP 2-MeSADP IDP	PPADS Reactive blue 2 Suramin 2-MeSAMP ARC67085MX ARC69931MX
$P2Y_{14}$	G_i (cAMP modulation)	UDP-glucose UDP-galactose	–

2-MeSADP: 2-Methylthioadenosine-5'-diphosphate. 2-MeSAMP: 2-Methylthioadenosine-5'-monophosphate. 2-MeSATP: 2-Methylthioadenosine-5'-triphosphate. ADP: adenosine diphosphate. AMP: adenosine monophosphate. ARC69931MX: N^6-[2-(Methylthio)-ethyl]-2-(3,3,3-trofluoropropyl)thio-5'adenylic acid. ARC67085MX: 2-Propylthio-D-β-γ-dichloromethylene-ATP. MRS2211. ATP: adenosine triphosphate. BzATP: benzyl-ATP. CTP: cytidine 5'-triphosphate. G_i: inhibitory G protein. Gq:. Group 1= high affinity for ATP, EC50=1μM, rapidly desensitisation. Group 2= lower affinity for ATP, EC50=10μM, slow desensitisation. Group 3= very low affinity for ATP, EC50=300-400μM, little desensitisation. G_s: stimulatory G protein. GTP: guanosine 5'triphosphate. INS365: Diuridine tetraphosphate. INS37217: P(1)-(Uridine 5')-P(4)-(2'-deoxycytidine 5')tetraphosphate tetrasodium salt. IsoPPADS: Pyroxidal-5-phosphate-6-azophenyl-2'5'-disulphonic acid. MRS2159: Pyroxidal-5-phosphate-6-azophenyl-4'-carboxylate. MRS2179: 2'deoxy-N^6-methyl-2-chloro-2'-deoxyadenosine-3'5'-biphosphate. MRS2279: (N)-Methanocarba-N^6-methyl-2-chloro-2'deoxyadenosine-3'5'-biphosphate. MRS2365: (N)-Methanocarba-2- Methylthioadenosine-5'-diphosphate. MRS2500: (N)-Methanocarba-N^6-Methyl-2-iodo-2'deoxyadenosine-3'5'-biphosphate. MRS2578. 1,4-di-(Phenylthioreido)butane. NF023: 8'8'-(Carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-mapthalenetrisulphonic acid). PPADS: Pyridoxal-5-phosphate-6-azophenyl-2',4'-disulphonic acid. TNP: 2'(3')-O(2,4,6-trinitrophenyl). UDP: uridine diphosphate. UMP: uridine monophosphate. UTP: uridine triphosphate. α-β-MeATP: α-β-methylene-adenosine-5'-triphosphate

Table 4.1. P2 receptor pharmacology (Adapted from Burnstock and Williams (2000), Brunschweiger and Muller (2006) and SigmaAldrich (2007)).

4.2.2.2 $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors

In addition to the $P2Y_2$ receptor, the human $P2Y_4$ and $P2Y_6$ receptors are sensitive to UTP and UDP. There is also limited evidence for UTP activity at $P2Y_{11}$ receptors (White *et al* 2003). The $P2Y_4$ receptor has been cloned from mouse (Suarez-Huerta *et al* 2001), human placenta (Communi *et al* 1997) and rat (Webb *et al* 1998). It shows restricted distribution compared to the $P2Y_2$ receptor, but is found in the placenta and expressed at low levels in the lung and vascular smooth muscle (Brunschweiger and Muller 2006). However, the $P2Y_4$ receptor appears to demonstrate species-specific agonist and antagonist pharmacology: ATP functions as a $P2Y_4$ receptor antagonist in humans but as a $P2Y_4$ receptor agonist in rat (Kennedy *et al* 2000). The $P2Y_6$ receptor has been cloned from both human (Communi *et al* 1996) and rat (Chang *et al* 1995). It is abundantly distributed throughout the body and is found associated with $P2Y_2$ receptors in the lung (Brunschweiger and Muller 2006). The $P2Y_{11}$ receptor has been cloned from human placenta, but has limited distribution and does not appear to be expressed in lungs (Communi *et al* 1997).

4.2.2.3 ATP and UTP concentrations in the airways

In the airways, epithelial cells constitutively release ATP and UTP (Donaldson *et al* 2000). To function as airway signalling molecules, ATP and UTP must be present in the airway surface liquid (ASL) at sufficient concentrations for the stimulation of purinergic receptors. The concentrations of ATP and UTP in ASL differ depending on the site in the airways. ATP concentrations in ASL range from 10-500nM, whilst UTP concentrations average ~40nM (Donaldson *et al* 2000, Okada *et al* 2006). ATP appears to be present at higher concentrations than UTP, mirroring their relative intracellular concentrations (Donaldson *et al* 2000). As discussed earlier, UTP and ATP are released from cells at a basal level, but secretion may also be stimulated. Lazarowski and Harden (1999) have demonstrated secretion of UTP and ATP from several cell types including airway epithelial cells in response to mechanical stimulus. Okada *et al* (2006) revealed a 1000 fold increase in ATP release from a human bronchial epithelial cell line triggered by hypotonic challenge.

4.2.2.4 Purinergic and pyrimidinergic effects in the airways

Numerous responses in the airways are mediated via purinergic signalling, including increased goblet cell-associated mucus secretion, increased ASL, increased mucus clearance and increased surfactant release.

4.2.2.4.1 Increased goblet cell-associated mucus secretion

Several groups have observed nucleotide triphosphate-mediated release of mucin from goblet cells (Conway *et al* 2003, Murakami *et al* 2003). The equal potency of ATP and UTP to stimulate mucin release in addition to inhibition by the P2 receptor antagonist suramin has supported the theory of $P2Y_2$ receptor involvement in nucleotide mediated goblet cell mucin release (Murakami *et al* 2003). However, nucleotide diphosphate-mediated goblet cell mucin release has also been demonstrated. Chen *et al* (2001) investigated the rank potencies of nucleotide triphosphates and diphosphates on goblet cell mucin secretion in human airway epithelial cells. Whilst less potent than their triphosphate counterparts, UDP and ADP both stimulated goblet cell mucin release. Adenosine also appeared to stimulate goblet cell mucin secretion but was the least potent of all the agonists tested. However mucin secretion following the addition of nucleotide to the airway epithelia appears to be short lived (up to 1hr), probably due to the rapid metabolism of ATP and UTP by a large number of ectoenzymes present in the airways (Larsen *et al* 2000, Tarran *et al* 2004).

4.2.2.4.2 Increased mucociliary clearance (MCC)

MCC is an airway defence mechanism involving the cilia-facilitated movement of mucus from the airways and is essential for the protection and maintenance of healthy lungs. Effective MCC is dependent on several factors, including the physical properties of mucus, sufficient sol layer volume, functioning cilia and efficient cilia beat frequency (CBF). Nucleotide activation of airway P2 receptors may have significant effects on the airway mucus clearance via the regulation of both airway surface liquid (ASL) and CBF. ASL consists of an overlying mucus layer (~55μm in thickness), which functions to trap particles and bacteria in the airways, and a sol layer (7μm in thickness), which maintains an optimum distance between the mucus layer and the epithelium (Jayaraman *et al* 2001, Krouse 2001). Optimal sol layer/ASL is essential to allow effective

movement of cilia in order to aid MCC and is determined by epithelial ionic secretion or absorption and subsequent osmotic movement of water across the epithelium. Research by Larsen *et al* (2000) established increased ASL volume following the addition of nucleotides onto airway epithelium, a possible consequence of purinergic receptor activation. Both $P2Y_2$ and P2X receptor activation stimulates increased intracellular calcium concentrations via phospholipase-induced mobilisation of intracellular calcium stores and direct influx of Ca^{2+} from extracellular stores via Ca^{2+} channels, respectively. Increased intracellular calcium concentrations mediate the activation of apical calcium-activated chloride channels, resulting in ion transport into the airway lumen. This can result in the osmotic movement of water into the airway and subsequent increases in respiratory tract liquid volume. Furthermore, purinergic agonists can induce attenuation of Na^+ uptake into epithelial cells (Inglis *et al* 1999) and cystic fibrosis transmembrane regulator (CFTR)-mediated chloride secretion into the ASL (Schweibert *et al* 1995) which may also have a regulatory effect on ASL volume.

In addition to the maintenance of optimal ASL volume, effective MCC is also dependent on ciliary beat frequency (CBF). It is likely that increases in CBF may be regulated by the activation of purinergic receptors in the airways, both directly and indirectly. Upregulation of CBF parallels increases in ASL volume and therefore may be a secondary response to ASL increases following nucleotide airway administration (Larsen *et al* 2000). Alternatively, research by Korngreen and Priel (1996) demonstrated a direct upregulation of CBF due to ATP-induced increases in intracellular calcium concentrations within the cilia themselves. Additionally, upregulation of ciliary activity in a concentration-dependent manner via ATP- or UTP-mediated activation of the $P2Y_2$ receptor, and possibly via the activation of the $P2Y_6$ receptor has been demonstrated in human nasal epithelial cells (Morse *et al* 2001). However, upregulation of CBF may also be stimulated by the pharmacologically active metabolite of ATP, adenosine. Adenosine appears to stimulate increases in CBF in both canine trachea and human HNE cells (Wong and Yeates 1992, Morse *et al* 2001). However, adenosine reduced CBF in rabbit trachea (Tamoaki *et al* 1989), suggesting possible species-dependent differences in the regulation of CBF by adenosine. Finally, airway mucus clearance during cough may also be increased subsequent to application of UTP into the airways (Noone *et al* 1999).

4.2.2.4.3 Increased surfactant release

UTP and ATP stimulate equipotent activation of $P2Y_2$ receptors on alveolar cells, resulting in surfactant release by type II alveolar cells (Gobran *et al* 1994).

4.2.2.4.4 Upregulation of MUC gene expression

In addition to its role as a secretagogue, UTP, but not ATP, ADP or UDP, can upregulate mucin gene expression in mice airways (Chen *et al* 2001). However upregulation of mucin gene expression appeared to be MAPK dependent, unlike the signalling pathway which is responsible for stimulating mucin release (Chen *et al* 2001), suggesting that regulation of mucin gene expression is controlled via an alternative receptor to $P2Y_2$ receptor such as the $P2Y_4$ or $P2Y_6$ receptor.

4.2.3 METABOLISM OF EXTRACELLULAR NUCLEOTIDES

The extracellular catabolism of nucleotides is mediated by a collection of ecto-enzymes including ectonucleotidase enzymes and ectophosphatases. These enzymes have been extensively investigated and were reviewed in the Second International Workshop on Ecto-ATPase and Related Ectonucleotidases (Zimmmerman *et al* 2000). They are responsible for the degradation of nucleotide triphosphates (NTPs) (UTP and ATP) to di-phosphates (NDPs) (UDP and ADP), mono-phosphates (NMPs) (UMP and AMP) and finally nucleosides (uridine and adenosine), and therefore control the relative concentrations of nucleotides and nucleosides in the ASL. Ectoenzymes are located bound to the apical epithelial membrane and are also secreted into the ASL. The substrates and resulting metabolites are specific for different ecto-enzyme subtypes. For example, ecto-apyrases and ecto-ATPases cleave phosphates of NTP, resulting in the metabolism of NTP into NDP and NMP, whereas ecto-5'nucleotidases convert NMP into the relative nucleoside and phosphate group and ecto-nucleotide pyrophosphatases cleave NTPs and NDPs into NMPs.

The metabolism of nucleotides by ecto-enzymes is relatively fast. ATP added to the surface of human nasal epithelial cells can be metabolised by ectoenzymes with a half-life of 5mins (Morse *et al* 2001). However, the enzyme-catalysed degradation of nucleotides is coupled with the formation of breakdown nucleotides and nucleosides, which can also be pharmacologically active. UDP and ADP are pharmacologically

active at P2 receptors, while adenosine has been shown to stimulate cardiovascular, bronchial and nervous system effects via P1 receptors. There is however little evidence for any pharmacological effects of uracil.

In addition to the metabolism of nucleotides, the reverse reaction, resulting in the formation of nucleotides can be catalysed by another group of ecto-enzymes. For example adenylate kinase, catalyses the phosphorylation of NMPs and NDPs into NDPs and NTPs respectively and nucleoside monophospho- and diphosphokinases converts 2 molecules of NMPs or NDP into NDP and adenosine or NTP and NMP respectively.

4.2.4 $P2Y_2$ RECEPTOR-SELECTIVE AGONISTS AND ANTAGONISTS

4.2.4.1 $P2Y_2$ receptor agonists

The identification of UTP as being therapeutically effective in the enhancement of MCC was extremely encouraging for the field of cystic fibrosis pharmacology. This led to increased investigation into the development of more stable or selective $P2Y_2$ receptor agonists in several laboratories. New classes of $P2Y_2$ receptor agonists, all of which are analogues of UTP and ATP substituted in the phosphate chain, maintain agonist properties whilst being considerably more stable than UTP or ATP. Inspire Pharmaceuticals have developed and patented several drug candidates (including INS316 and INS365), all stable analogues of UTP, for the treatment of dry eye, cystic fibrosis and retinal disease. Research into the modelling of the $P2Y_2$ receptor and design of selective and stable $P2Y_2$ receptor agonists is extensive and ongoing (Ivanov *et al* 2007). However, although the development of such agonists is encouraging, no stable or selective $P2Y_2$ receptor agonists are currently commercially available. The absence of such agonists on the commercial market has made purinergic receptor research problematic.

4.2.4.2 $P2Y_2$ receptor antagonists

The development of specific $P2Y_2$ receptor antagonists has also proved difficult. Several P2 receptor antagonists have been identified including PPADS, DIDS, suramin and dyes such as reactive blue-2. However, none of these antagonists are specific for any one P2 receptor subtype. Newer P2 receptor antagonists include TNP-GTP (2'(3')-O(2,4,6-trinitrophenyl)guanosine 5'triphosphate) ($P2X_1$ and $P2X_3$), MRS2216 (2-

chloro-2'-deoxy-N^6-methyladenosine-3'5'-bisphosphate) ($P2Y_1$), AR-C 69931 MX (N6-(2-methylthioethyl)-2-(3,3,3,-trifluoropropylthio)-β-γ-dichloromethylene ATP) ($P2Y_{12}$) and Brilliant Blue G ($P2X_7$). (See Table 4.1 for P2 receptor antagonists). However, despite intense investigation, there are still very few P2 receptor antagonists, particularly specific $P2Y_2$ receptor antagonists, reliable for receptor subtype characterisations and receptor research.

Suramin is currently the predominant compound used for the antagonism of $P2Y_2$ receptors. It is an extremely stable polysulphated napthylurea developed by Oskar Dressel in Germany in 1916. Since then, suramin has been implicated in the treatment of several diseases. Suramin has the capacity to inhibit several enzymes including reverse transcriptase (De Celerq 1987), DNA and RNA polymerases (Ono *et al* 1998), topoisomerases (Bojinowski *et al* 1992), heparinase (Nakajima *et al* 1991) and several growth factors, thus exerting a variety of effects including antiviral (De Celerq 1987), antiparasitic, antineoplastic, antiproliferative and antiangiogenetic properties. However, due to its stability and long half-life (>30days), suramin is extremely toxic leading to mucopolysaccharidosis-like pathologic conditions, thus limiting its therapeutic usage. For the chemical structure of suramin see Fig. 4.1i.

4.3 AIMS AND OBJECTIVES

HYPOTHESIS. *In a chronically OA challenged guinea pig, nebulised exposures of the secretagogues, UTP, UDP and ATP, induce goblet cell degranulation and mucus secretion, which leads to airway mucus accumulation and ultimately a reduction in lung function.*

4.3.1 AIM

The aim of this chapter was to utilise plethysmography and histological methods to analyse the effect of the purinergic receptor agonists UTP, UDP and ATP on goblet cell-associated mucin secretion and changes in lung function.

4.3.2 OBJECTIVES

- To demonstrate possible changes in lung function measurements subsequent to increasing doses of a single nebulised UTP, UDP or ATP exposure in chronically OA challenged guinea pigs.

- To identify the effect of the P2 receptor antagonist suramin on possible changes in lung function measurements following UTP exposure.

- To demonstrate possible changes in the mean % of AB/PAS-positive bronchiolar epithelial area subsequent to nebulised UTP, UDP or ATP exposure in chronically OA challenged guinea pigs.

- To identify the effect of the P2 receptor antagonist suramin on possible changes in the mean % of AB/PAS-positive bronchiolar epithelial area following nebulised UTP exposures.

4.4 METHODS

Groups of 6 male Dunkin-Hartley guinea pigs (supplied by Harlan, UK) weighing between 200-250g were used for all protocols.

4.4.1 SENSITISATION

Animals were sensitised on days 1 and 5 with an i.p, bilateral injection of a suspension containing 100 μg of OA and 100 mg aluminium hydroxide.

4.4.2 NEBULISED OVALBUMIN CHALLENGES

14 days subsequent to the sensitisation period (day 15), guinea pigs were challenged with either an acute OA challenge or chronic OA challenge. For all OA challenges, a Wright nebuliser was used to supply air at a pressure of 20p.s.i. and at a rate of 0.3ml/min into a sealed stainless steel exposure chamber (40cm diameter, 15cm height). If any animal appeared in distress, the animal was removed from the exposure chamber and challenge considered complete.

4.4.2.1 Acute OA challenge

14 days following sensitisation (day 15), animals were exposed to a nebulised solution of low dose OA (0.01% for 1hr).

4.4.2.2 Chronic OA challenge

14 days following sensitisation (day 15), animals were exposed to a single nebulised solution of low dose OA (0.01% for 1hr). Animals were subsequently exposed to a nebulised solution of high dose OA (0.1% for 1hr) on days 17, 19, 21, 23, 25, 27 and 29. Mepyramine (30mg/kg) was administered by bilateral, i.p. injection 30mins prior to UTP exposure on days 17, 19, 21, 23, 25 and 27.

4.4.3 EXPOSURES TO INDUCE MUCUS SECRETION

Subsequent to acute OA challenge or chronic OA challenge, guinea pigs were exposed to the following nebulised solutions to potentially induce goblet cell associated mucin secretion: 1) a single UTP exposure (1mM or 10mM for 15mins) 22hrs 45mins following chronic OA challenge or acute OA challenge 2) a single UDP exposure (10mM for 15mins) 22hrs 45mins following chronic OA challenge or 3) a single ATP exposure (3mM for 1min) 23hrs following chronic OA challenge. Lung function responses were measured immediately prior to exposures and at 0, 5, 10, 15mins and every 15mins up to 1hr subsequent to exposures. For all exposures, a Wright nebuliser was used to supply air at a pressure of 20p.s.i. and at a rate of 0.3ml/min into a sealed perspex chamber (15x 15x 32cm).

4.4.3.1 Antagonists

Suramin dissolved in saline (60mg/kg) or vehicle was administered by i.p., bilateral injection 30mins prior to UTP exposure.

4.4.4 HISTAMINE-MEDIATED BRONCHOCONSTRICTION

To assess bronchoconstriction to histamine (as previously described in Chapter 2), animals were exposed to a single nose-only nebulised solution of a threshold dose of histamine (1mM for 20secs) 24hrs subsequent to chronic OA challenge. Lung function responses were measured immediately prior to histamine exposure and 0, 5 and 10mins subsequent to histamine exposure.

4.4.5 LUNG FUNCTION MEASUREMENTS

Whole body plethysmography was used to measure specific airway conductance (sG_{aw}) as previously described (Chapter 2).

4.4.6 TOTAL AND DIFFERENTIAL CELL COUNTS

24hrs subsequent to acute OA challenge or chronic OA challenge (1hr subsequent to UTP, UDP or ATP exposure) animals were terminated by a lethal overdose of sodium

pentobarbitone and lungs lavaged. Total cells and differential cell counts (per ml of lavage fluid) were determined using a Neubauer haemocytometer and cytospin spears, as previously described (Chapter 2).

4.4.7 HISTOLOGICAL ANALYSIS OF GUINEA PIG LUNGS

Immediately following BAL, lungs were removed from the thoracic cavity and fixed with formaldehyde. 3-5mm tangentially sliced portions of lung were processed into wax blocks, sectioned (3μm) using a Leica microtome and fixed onto glass slides. Slides were stained with AB/PAS and Mayers haemolum and each bronchiole analysed to give the % of AB/PAS-positive area of the bronchiolar epithelium. This was calculated for each bronchiole and mean values calculated. Detailed methodology is described in Chapter 2.

4.5 RESULTS

4.5.1 UTP

The effect of nebulised UTP exposures in chronically OA treated guinea pigs on lung function responses, total and differential cell counts in BALF and goblet cell associated mucin accumulation was assessed.

4.5.1.1 Effect of UTP exposure on lung function

Fig. 4.2 represents the mean time course for changes in sG_{aw} following a single nebulised UTP exposure (1mM for 15mins) or vehicle exposure (15mins) in sensitised guinea pigs, 24hrs subsequent to chronic OA challenge. In sensitised, chronically OA challenged guinea pigs, a single nebulised UTP exposure stimulated a gradual fall in lung function, identified as a continuous reduction in sG_{aw} up to 1hr and significant at 30mins and 1hr subsequent to UTP exposure, compared to vehicle. A single nebulised UTP exposure stimulated no significant reduction in sG_{aw} in sensitised, unchallenged guinea pigs.

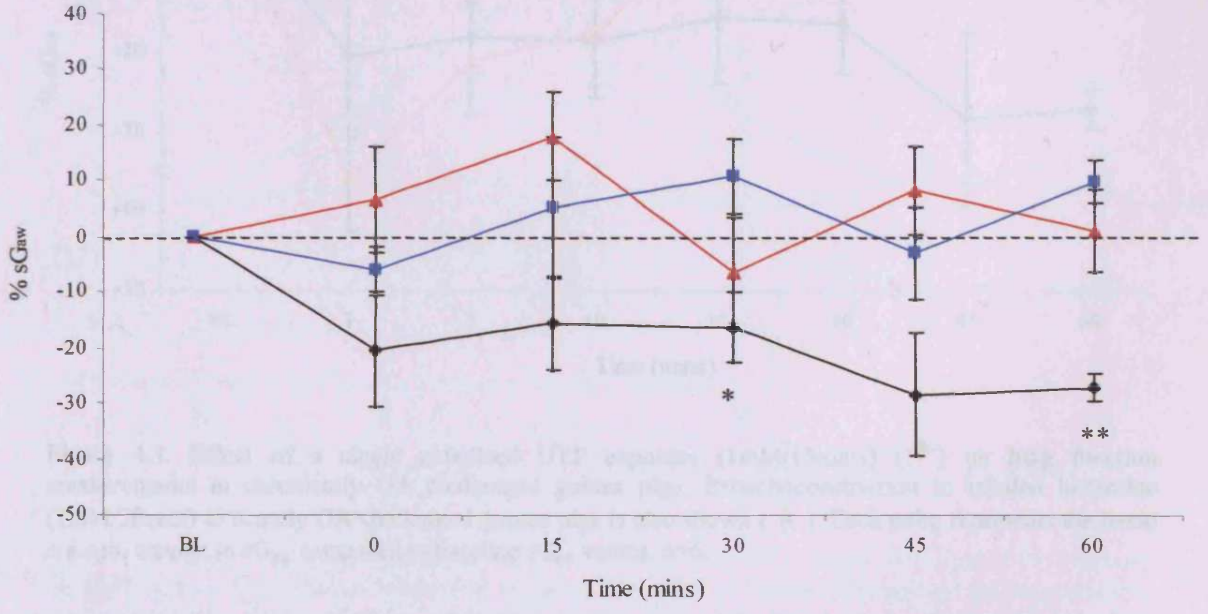


Figure 4.2. The effect of a single UTP exposure (1mM/15mins) (◆) or single nebulised vehicle (15mins) (■) on lung function measurements in chronically OA challenged guinea pigs. The effect of a single UTP exposure (1mM/15mins) (▲) on lung function measurements in sensitised, non-OA challenged guinea pigs is also shown. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different to vehicle.

4.5.1.2 Comparison of lung function responses following histamine or UTP exposure

Fig. 4.3 represents the mean time course for the changes in sG_{aw} following a single UTP exposure (1mM for 15mins) in chronically OA challenged guinea pigs, compared to a typical bronchoconstriction to histamine (1mM for 20secs) in acutely OA challenged guinea pigs. A single nose-only histamine exposure of 1mM (20secs) is used as a threshold dose of histamine, which induces bronchoconstriction in sensitised and challenged guinea pigs but not in naïve animals and is therefore utilised as a measure of airway hyperreactivity (AHR). A single nebulised histamine exposure (1mM for 20secs) stimulated an immediate bronchoconstriction (-35.9 ± 6.3) which recovered rapidly (at 15mins subsequent to histamine exposure). However, a gradual reduction in lung function, persistent up to 1hr, was induced in chronically OA challenged guinea pigs following a single nebulised UTP exposure.

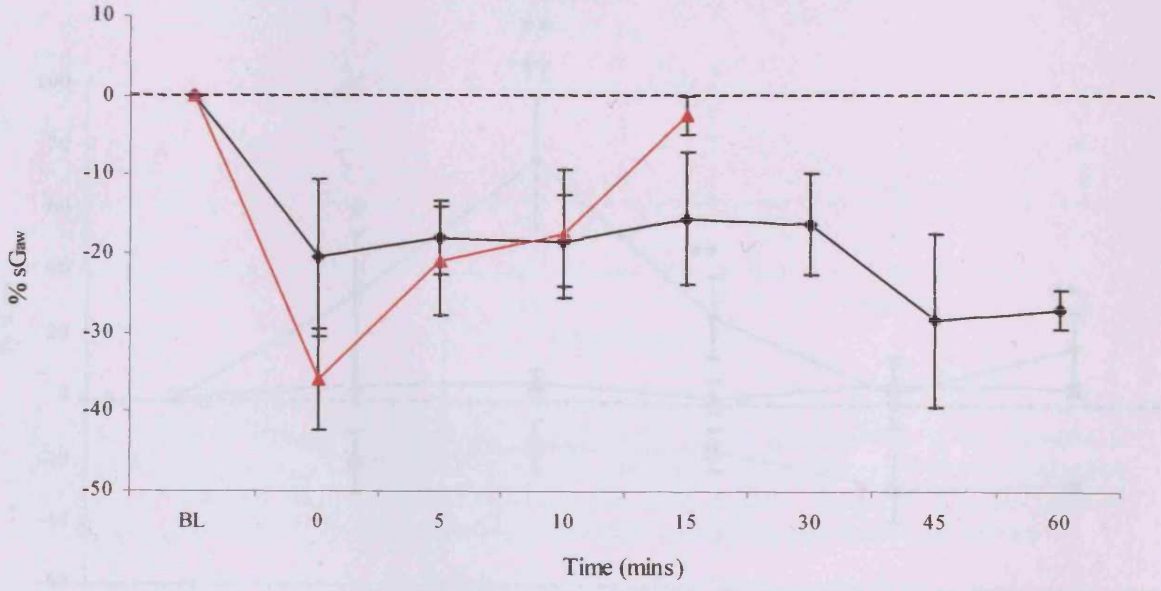


Figure 4.3. Effect of a single nebulised UTP exposure (1mM/15mins) (◆) on lung function measurements in chronically OA challenged guinea pigs. Bronchoconstriction to inhaled histamine (1mM/20secs) in acutely OA challenged guinea pigs is also shown (▲). Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6.

4.5.1.3 Effect of suramin on lung function responses to UTP exposure

In this study, the effect of suramin on UTP-induced lung function responses in chronically OA challenged guinea pigs was assessed. In chronically OA challenged guinea pigs, a single nebulised UTP exposure (1mM for 15mins), induced a gradual reduction in sG_{aw} up to 60mins following exposure. However, pretreatment with an i.p. bilateral injection of suramin 30mins prior to nebulised UTP exposure, converted the UTP-induced reduction in lung function to a significant increase in sG_{aw} (+75.4 ± 21.1%), which recovered to baseline at 45mins. No significant change in sG_{aw} from baseline was revealed following a single nebulised vehicle exposure in chronically OA challenged guinea pigs, pretreated with a bilateral, i.p. injection of suramin (60mg/kg) 30mins prior to vehicle exposure (Fig. 4.4)

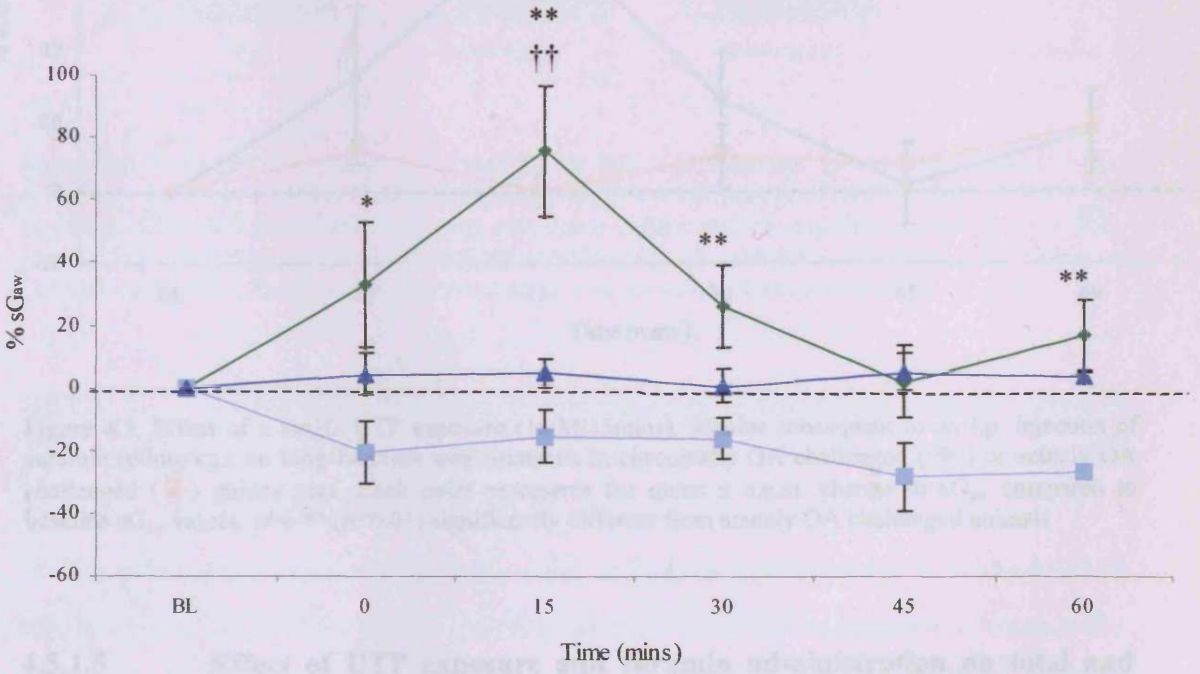


Figure 4.4. Effect of a single nebulised vehicle (15mins) (▲) or a single nebulised UTP exposure (1mM/15mins) (◆), 30mins subsequent to an i.p. injection of suramin (60mg/kg), on lung function measurements in chronically OA challenged guinea pigs. The effect of a single UTP exposure (1mM/15mins) on lung function measurements in non suramin-treated, chronically OA challenged guinea pigs (■) is also shown. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different to non-suramin treated guinea pigs. † ($p<0.05$) †† ($p<0.01$) significantly different to vehicle.

4.5.1.4 Effect of suramin on UTP-induced lung function responses in acutely OA challenged and chronically OA challenged guinea pigs

A single nebulised UTP exposure (1mM for 15mins), 30mins subsequent to an i.p. injection of suramin (60mg/kg), induced a significant increase in sG_{aw} (+75.4 ± 21.1%), which recovered to baseline at 45mins in chronically OA challenged guinea pigs. However in acutely OA challenged guinea pigs, a single UTP nebulised exposure (1mM for 15mins), 30mins subsequent to a bilateral i.p. injection of suramin (60mg/kg), induced no change in sG_{aw} from baseline sG_{aw} values (Fig. 4.5).

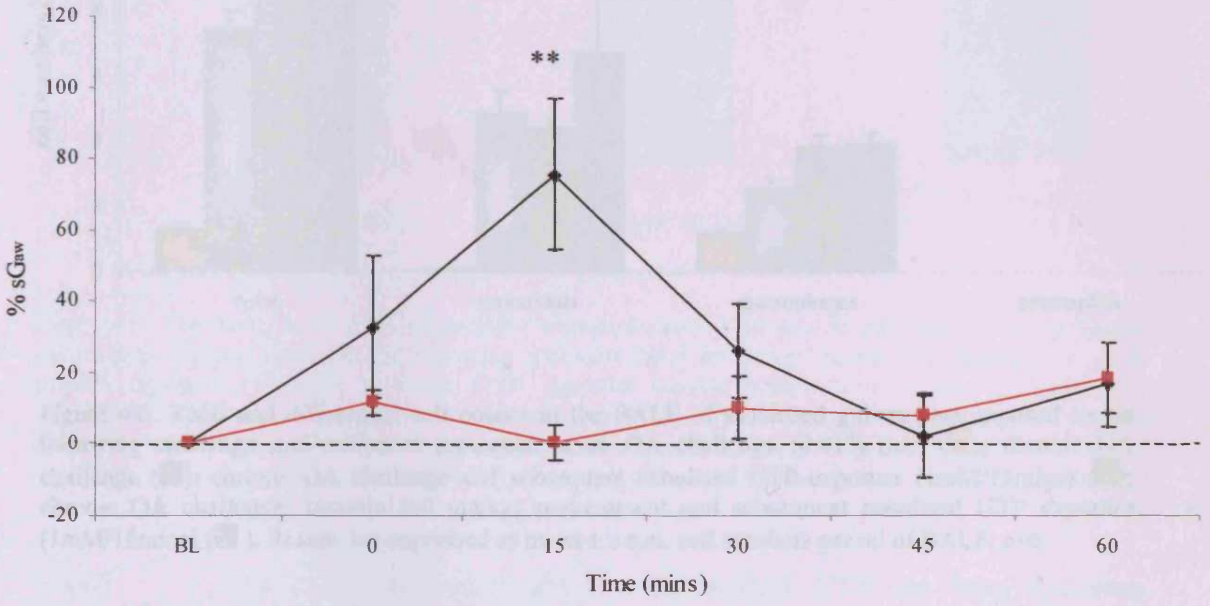


Figure 4.5. Effect of a single UTP exposure (1mM/15mins), 30mins subsequent to an i.p. injection of suramin (60mg/kg), on lung function measurements in chronically OA challenged (-◆-) or acutely OA challenged (-■-) guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6 ** ($p<0.01$) significantly different from acutely OA challenged animals

4.5.1.5 Effect of UTP exposure and suramin administration on total and differential cell counts in BALF

Fig. 4.6 represents the effect of a single nebulised UTP exposure, with and without suramin pretreatment on total and differential cell numbers per ml of BALF in chronically OA challenged guinea pigs. Inflammatory cell numbers in BALF were significantly increased in guinea pigs exposed to a chronic OA challenge, compared to

guinea pigs challenged with an acute OA challenge. However, BALF inflammatory cell numbers were unchanged following UTP nebulised exposure, with or without suramin pretreatment.

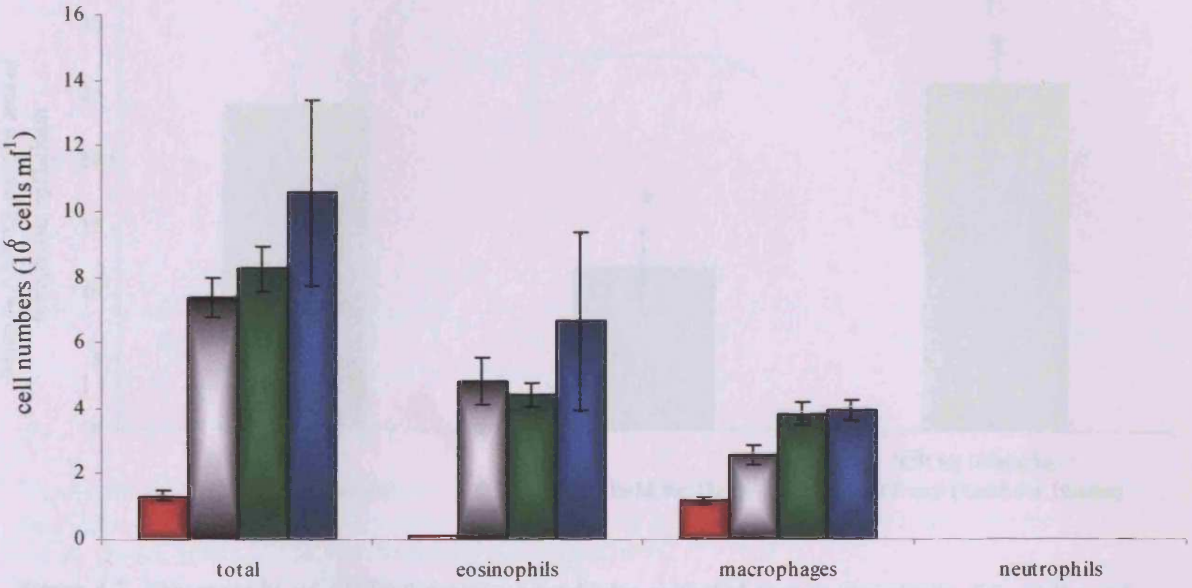


Figure 4.6. Total and differential cell counts in the BALF of sensitised guinea pigs exposed to the following challenge and treatment protocols: acute OA challenge (0.01%/1hr). ( ); chronic OA challenge ( ); chronic OA challenge and subsequent nebulised UTP exposure (1mM/15mins) ( ); chronic OA challenge, suramin (60 mg/kg) pretreatment and subsequent nebulised UTP exposure (1mM/15mins) ( ). Results are expressed as mean ± s.e.m. cell numbers per ml of BALF. n=6.

4.5.1.6 Effect of UTP exposures and suramin administration on the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs

In this part of the study, all animals were sensitised and exposed to a chronic OA challenge and lungs removed for histological analysis 24hrs subsequent to chronic OA challenge. AB/PAS stained sections of guinea pig left lung were analysed for the % of AB/PAS positive bronchiolar epithelial area. The mean % of AB/PAS-positive bronchiolar epithelial area was reduced in guinea pigs exposed to a single nebulised UTP exposure (1mM for 15mins) 22hrs 45mins subsequent to chronic OA challenge, compared to guinea pigs exposed to a vehicle exposure (15mins) 22hrs 45mins subsequent to chronic OA challenge. However, the UTP-induced reduction in mean %

of AB/PAS-positive bronchiolar epithelial area was inhibited by a single i.p. injection of suramin (60mg/kg) 30mins prior to UTP exposure (Fig. 4.7).

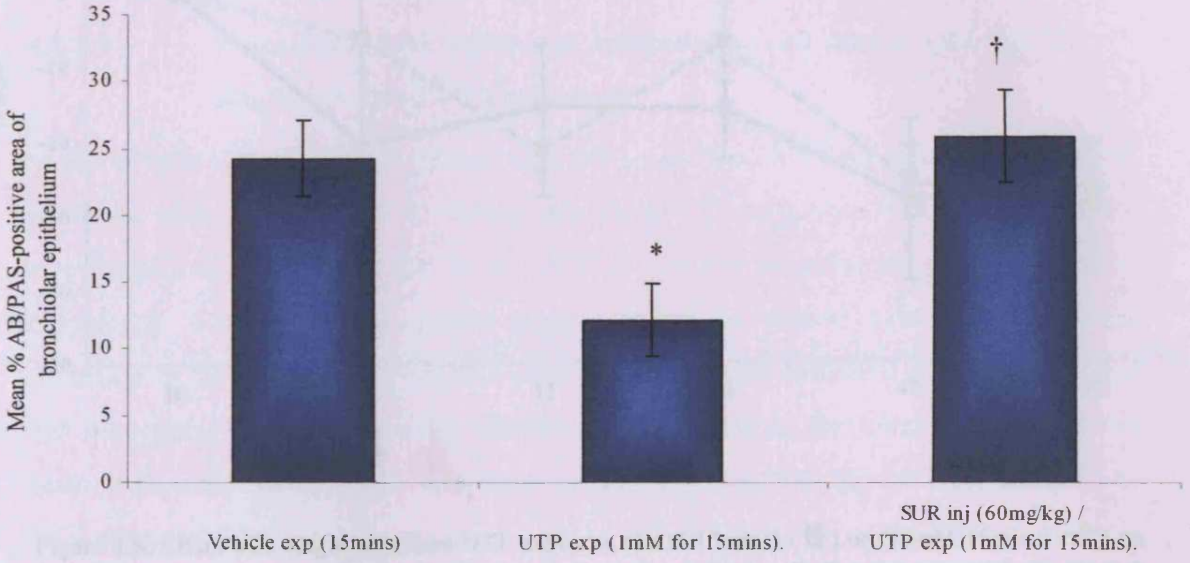


Figure 4.7. The mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs exposed to one of the following protocols 24hrs following chronic OA challenge: a single vehicle exposure (15mins); a single UTP exposure (1mM/15mins) or a single UTP exposure (1mM/15mins) 30mins subsequent to an i.p. injection of suramin (60mg/kg). Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of guinea pig left lung. n=5. * ($p<0.05$) significantly different to vehicle. † ($p<0.05$) significantly different to UTP exposed animals.

4.5.1.7 Effect of increasing doses of nebulised UTP on lung function measurements

Fig. 4.8 represents the mean time course for the changes in sG_{aw} in chronically OA challenged guinea pigs following one of the following single nebulised UTP exposures: 1mM (15mins) or 10mM (15mins). Both concentrations of nebulised UTP stimulated a gradual reduction in sG_{aw} up to 1hr subsequent to UTP exposure.

4.5.1.8 Effect of increasing UTP exposure doses on epithelial stored mucin in chronically OA challenged guinea pigs

The mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs was significantly reduced by 69.5% following a single nebulised exposure of 1mM UTP (15mins) compared to vehicle. However, a single nebulised

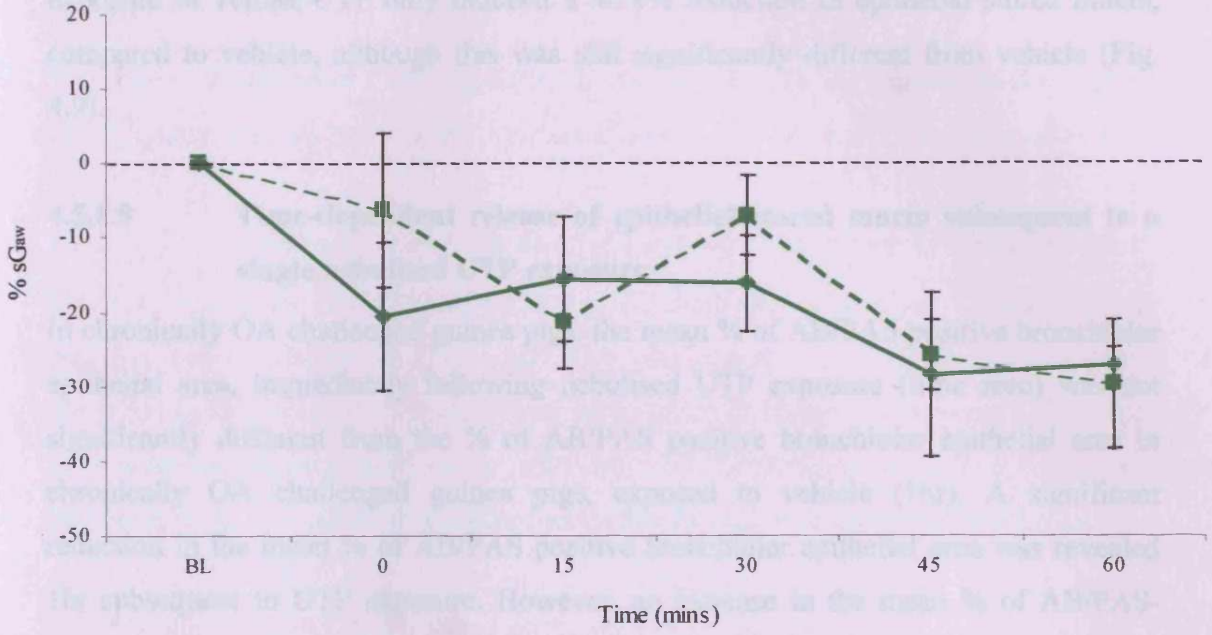


Figure 4.8. Effect of a single nebulised UTP exposure (1mM/15mins) (■) or (10mM/15mins) (◆) on lung function measurements in chronically OA challenged guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6.

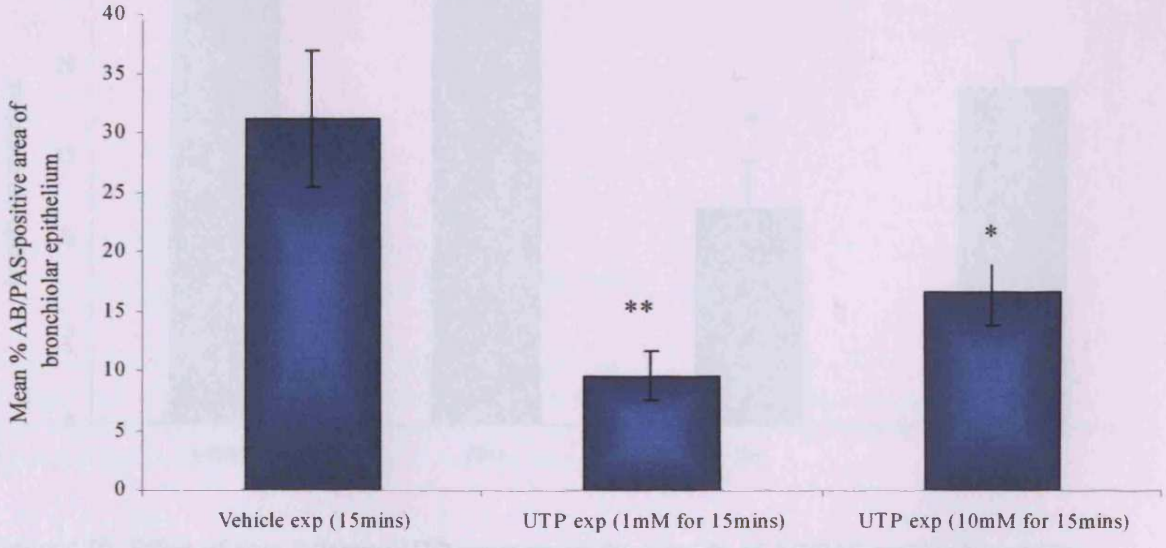


Figure 4.9. The mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs exposed with one of the following protocols 24hrs following chronic OA challenge: a single vehicle exposure (15mins); a single UTP exposure (1mM for 15mins) or a single UTP exposure (10mM for 15mins). Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of guinea pig left lung. n=6. *($p<0.05$) **($p<0.01$) significantly different from vehicle.

exposure of 10mM UTP only induced a 46.8% reduction in epithelial stored mucin, compared to vehicle, although this was still significantly different from vehicle (Fig. 4.9).

4.5.1.9 Time-dependent release of epithelial stored mucin subsequent to a single nebulised UTP exposure

In chronically OA challenged guinea pigs, the mean % of AB/PAS positive bronchiolar epithelial area, immediately following nebulised UTP exposure (time zero) was not significantly different from the % of AB/PAS positive bronchiolar epithelial area in chronically OA challenged guinea pigs, exposed to vehicle (1hr). A significant reduction in the mean % of AB/PAS positive bronchiolar epithelial area was revealed 1hr subsequent to UTP exposure. However, an increase in the mean % of AB/PAS-positive bronchiolar epithelial area was revealed between 1hr and 48hrs subsequent to UTP exposure (Fig. 4.10).

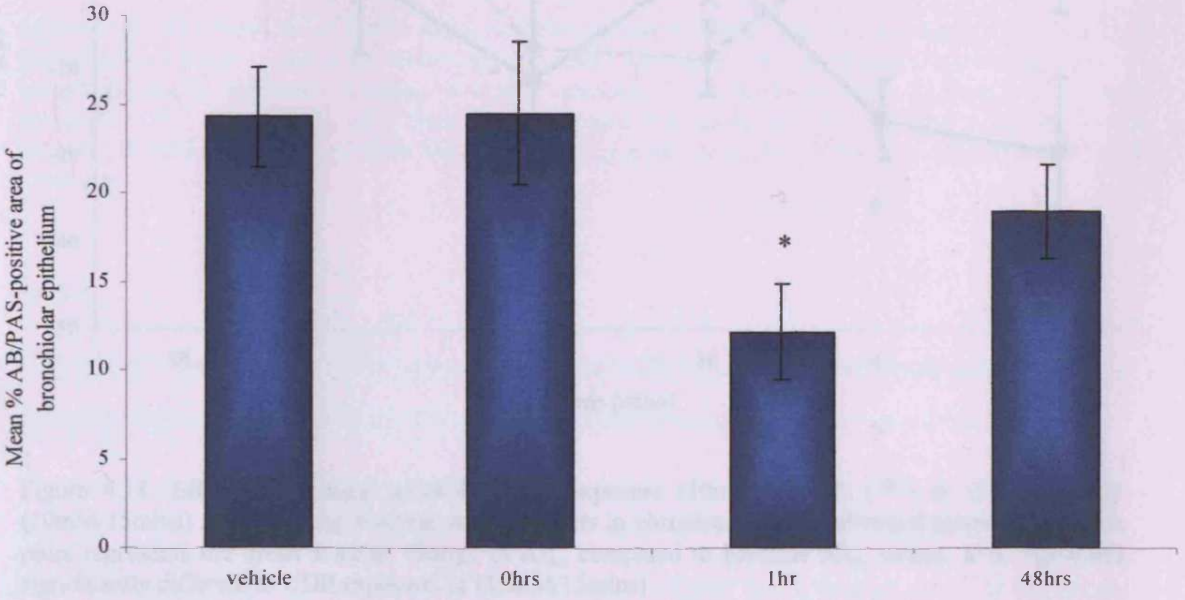


Figure 4.10. Effect of time following UTP exposure on the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs. Chronically OA challenged guinea pigs were treated with one of the following experimental protocols: a single UTP exposure (10mM for 15mins) and lungs removed at 0hrs, a single UTP exposure (10mM for 15mins) and lungs removed at 1hr, a single UTP exposure (10mM for 15mins) and lungs removed at 48hrs or a single vehicle exposure (15mins) and lungs removed at 1hr. Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of guinea pig left lung. n=6. * ($p<0.05$) significantly different to vehicle.

4.5.2 UDP

The effect of nebulised UDP exposures in chronically OA challenged guinea pigs on lung function responses and goblet cell associated mucin accumulation was assessed.

4.5.2.1 Comparison of UDP and UTP exposure on changes in lung function

Fig. 4.11 represents the effect of UDP or UTP exposure (10mM for 15mins) on lung function measurements in chronically OA challenged guinea pigs. A comparable reduction in lung function responses was revealed up to 30mins subsequent to UDP and UTP nebulised exposures. However, although the UTP-mediated reduction in sG_{aw} was persistent up to 1hr, the UDP-mediated reduction in sG_{aw} was recovered at 45mins.

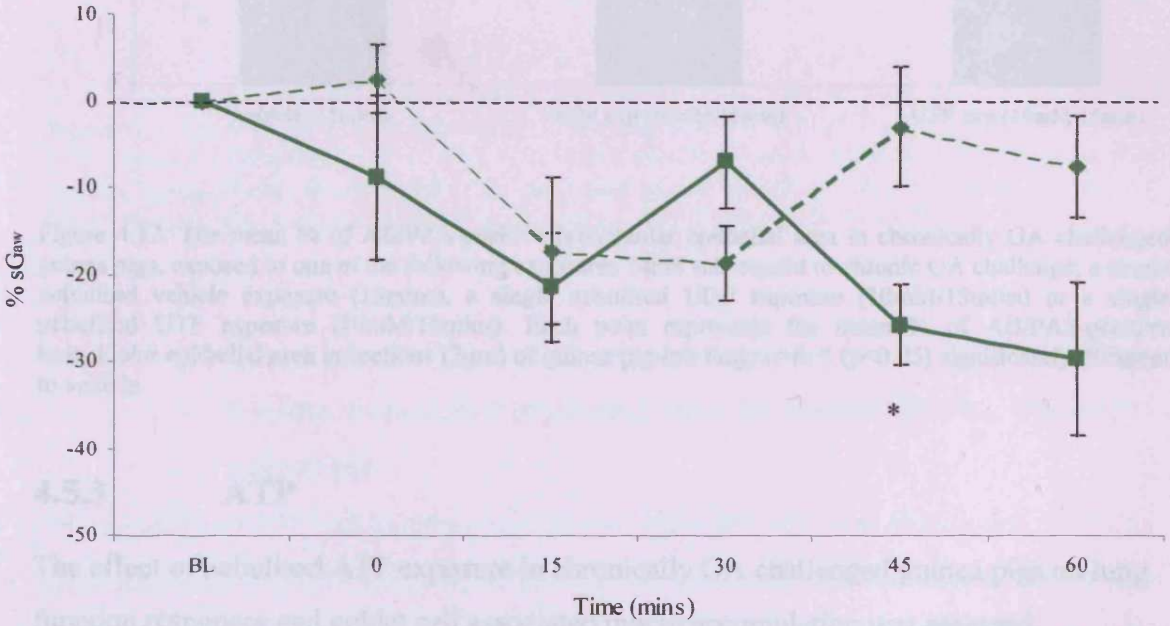


Figure 4.11. Effect of a single nebulised UDP exposure (10mM/15mins) (◆) or UTP exposure (10mM/15mins) (■) on lung function measurements in chronically OA challenged guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6. *($p<0.05$) significantly different to UDP exposure. or (10mM/15mins)

4.5.2.2 Effect of nebulised UDP exposure on the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs

The mean % of AB/PAS-positive bronchiolar epithelial area was significantly reduced following both UDP (10mM for 15mins) and UTP (10mM for 15mins) exposure (by

51.0% and 46.0% respectively) in chronically OA challenged guinea pigs compared to vehicle (Fig. 4.12).

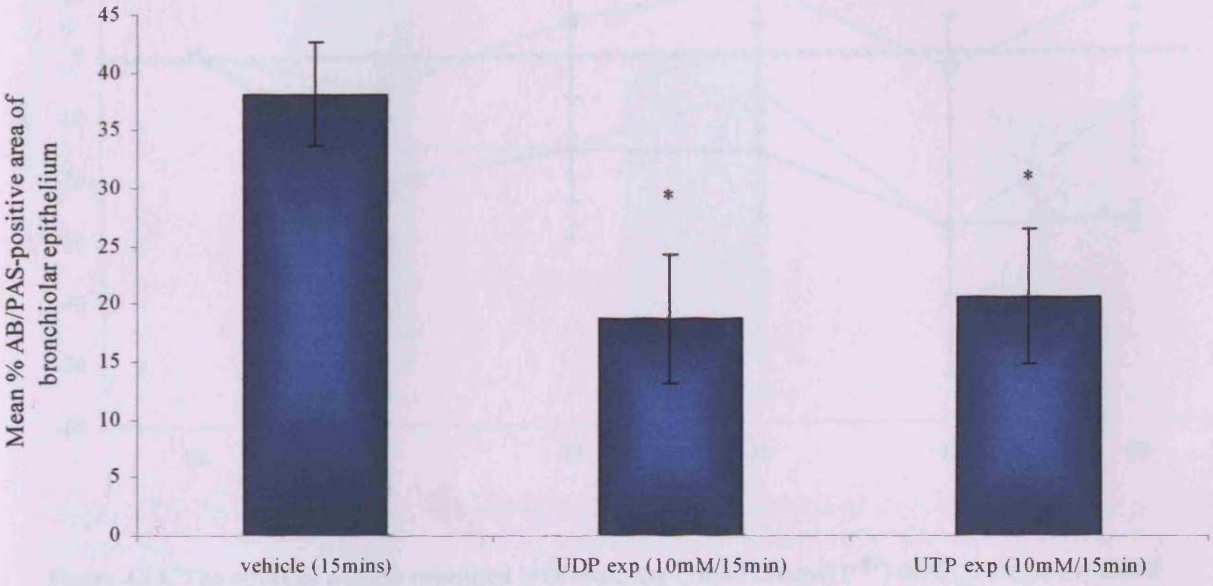


Figure 4.12. The mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs, exposed to one of the following exposures 24hrs subsequent to chronic OA challenge: a single nebulised vehicle exposure (15mins), a single nebulised UDP exposure (10mM/15mins) or a single nebulised UTP exposure (10mM/15mins). Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of guinea pig left lung. n=6. * ($p<0.05$) significantly different to vehicle.

4.5.3 ATP

The effect of nebulised ATP exposure in chronically OA challenged guinea pigs on lung function responses and goblet cell associated mucin accumulation was assessed.

4.5.3.1 Comparison of ATP and UTP exposure on changes in lung function

Fig. 4.13 represents the effect of UTP exposure (10mM for 15mins) or ATP exposure (3mM for 1min) on lung function measurements in chronically OA challenged guinea pigs. Despite a comparable reduction in lung function responses subsequent to ATP and UTP nebulised exposure, ATP revealed no significant reduction in sG_{aw} compared to vehicle exposure, possibly due to large interindividual differences.

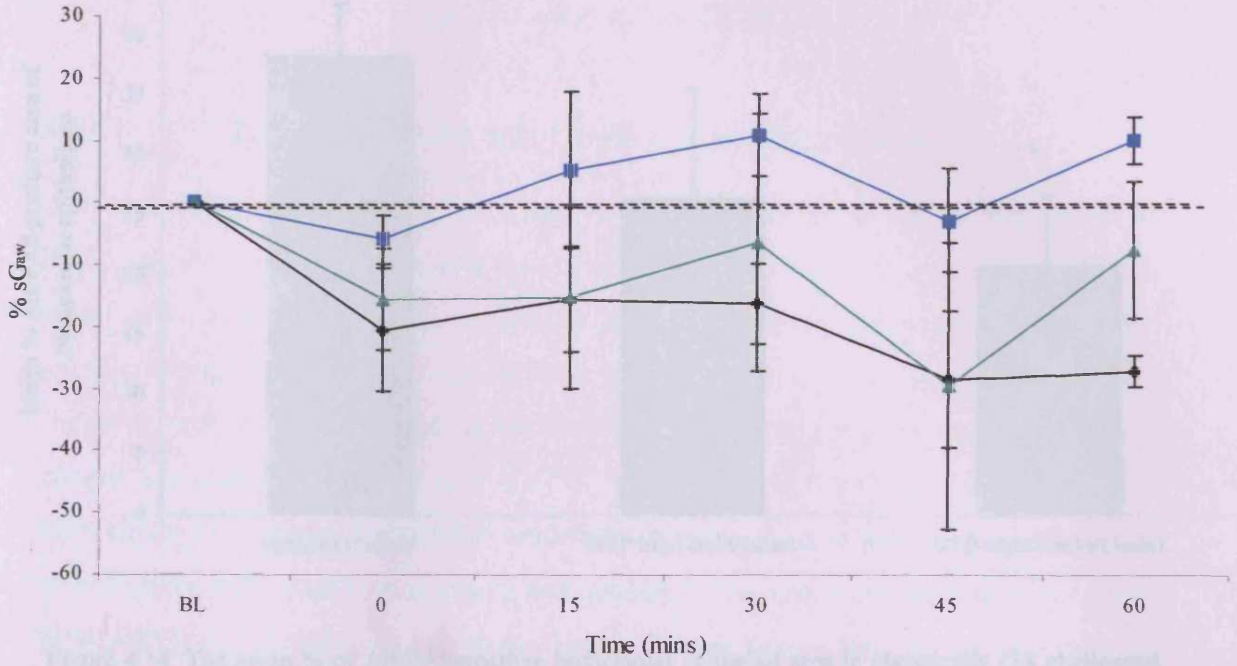


Figure 4.13. The effect of a single nebulised UTP exposure (1mM/15mins) (◆) (n=6), a single nebulised ATP exposure (3mM/1min) (▲) (n=4) or a single nebulised vehicle exposure (■) (n=6) on lung function measurements in chronically OA challenged guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. * ($p<0.05$) significantly different to vehicle.

4.5.3.2 Effect of nebulised ATP exposure on the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs

The mean % of AB/PAS-positive bronchiolar epithelial area was significantly reduced following UTP exposure (10mM for 15mins) in chronically OA challenged guinea pigs compared to vehicle. However, the mean % of AB/PAS-positive bronchiolar epithelial area was not significantly reduced following ATP (3mM for 1min) exposure (Fig. 4.14).

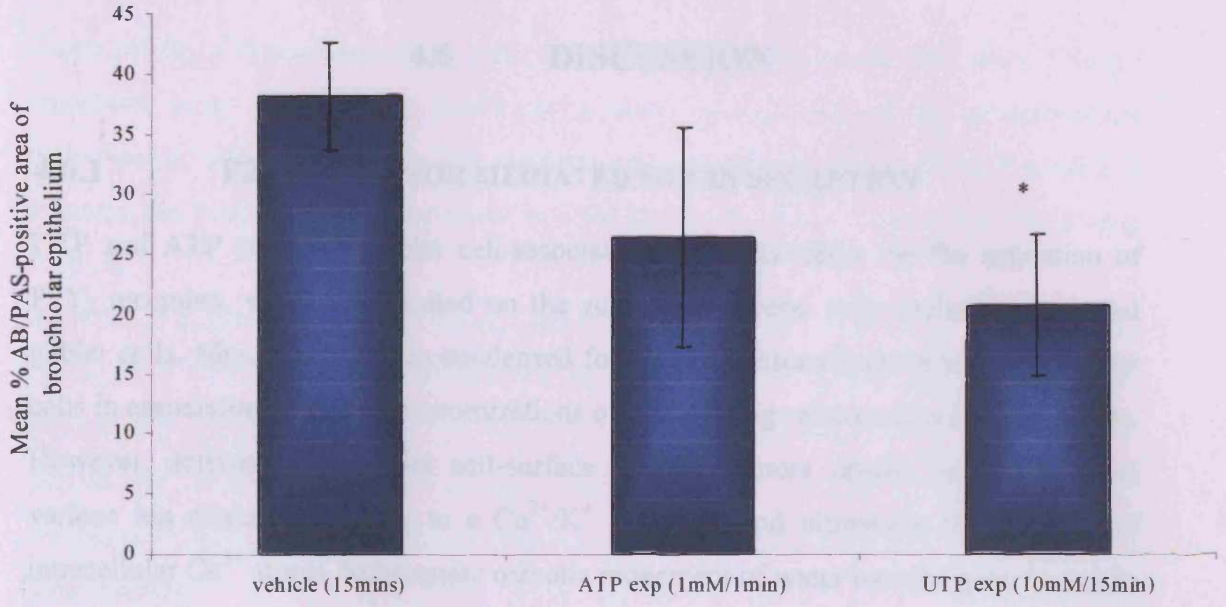


Figure 4.14. The mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs, exposed to one of the following exposures 24hrs subsequent to chronic OA challenge: a single nebulised vehicle exposure (15mins), a single nebulised UTP exposure (10mM/15mins) or a single nebulised ATP exposure (3mM/1min). Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of guinea pig left lung. n=6. * ($p<0.05$) significantly different to vehicle.

4.6 DISCUSSION

4.6.1 $P2Y_2$ RECEPTOR MEDIATED MUCIN SECRETION

UTP and ATP stimulate goblet cell-associated mucin secretion via the activation of $P2Y_2$ receptors, which are located on the surface of several cells including epithelial goblet cells. Mucin is stored in condensed form within intracellular granules of goblet cells in association with high concentrations of neutralising calcium ions (Rogers 1994). However, activation of goblet cell-surface $P2Y_2$ receptors results in activation of various ion channels, leading to a Ca^{2+}/K^+ exchange and ultimately mobilisation of intracellular Ca^{2+} stores. Subsequent osmotic movement of water into the granule results in hydrolysis and expansion of mucin and ultimately the exocytotic secretion of mucin from goblet cells into the airway lumen. This is thought to be a fast process, resulting in the discharge of large quantities of mucus in just tens of milliseconds (Rogers 1994).

4.6.2 UTP-INDUCED RESPONSES IN THE AIRWAYS OF CHRONICALLY OA CHALLENGED GUINEA PIGS

In chronically OA challenged guinea pigs, inhaled UTP (1mM for 15mins) revealed a gradual reduction in lung function, reaching a nadir at 45mins and persisting up to 1hr. It could be argued that the UTP-induced reduction in lung function may be a result of bronchoconstriction, stimulated directly by the activation of UTP-sensitive receptors on airway smooth muscle. Additionally, Schulman *et al* (1999) have previously demonstrated ATP and UTP-mediated mast cell degranulation via the activation of mast cell surface $P2Y_2$ receptors, raising the possibility that the UTP-mediated reduction in lung function in our model is a bronchoconstriction response mediated by H_1 receptor activation by mast cell-derived histamine. However, the results indicate that UTP is unlikely to stimulate bronchoconstriction in the guinea pig airway. Firstly, a single nebulised UTP exposure failed to induce a response in naive animals, suggesting that UTP had no direct effect on airway smooth muscle in normal guinea pig airways. Additionally, a single UTP exposure failed to stimulate bronchoconstriction in sensitised guinea pigs, which possess a viable mast cell population and it is therefore unlikely that the UTP-mediated reduction in lung function is mast cell-mediated.

Furthermore, a typical bronchoconstriction response, demonstrated following a single threshold dose of histamine (1mM for 20secs) is characterised by an immediate reduction in sG_{aw} and equally fast recovery. This markedly differs from the gradual reduction in lung function subsequent to UTP exposure, which is persistent for at least 60mins.

In addition to changes in lung function, histological analysis revealed a reduction in the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs, suggesting goblet cell mucin secretion. It is therefore proposed that in the guinea pig model of chronic allergen challenge, UTP induces goblet cell mucin secretion, resulting in mucus accumulation in the airways and a subsequent reduction in lung function.

4.6.3 UTP-INDUCED AIRWAY RESPONSES AND THE $P2Y_2$ RECEPTOR

Goblet cell-associated mucin secretion by UTP is mediated via the activation of $P2Y_2$ receptors (Conway *et al* 2003). Suramin is currently the predominant compound used for the antagonism of $P2Y_2$ receptors, but it is non-selective and therefore inhibits additional P2 receptors. Pretreatment with suramin (60mg/kg), 30mins prior to UTP exposure, inhibited the UTP-induced reduction in lung function and attenuated the UTP-induced reduction in the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs, thus providing evidence of P2 receptor mediated mucin secretion following UTP exposure.

However, in chronically OA challenged guinea pigs, a single UTP exposure 30mins subsequent to an i.p. injection of suramin, blocked the slow onset reduction in sG_{aw} and induced a substantial increase in sG_{aw}, significant at 30mins and recovered at 1hr post exposure. This would imply a bronchodilator action. It is unclear which precise mechanisms are involved in this response. Suramin is extremely stable, possesses a half-life of greater than 30days and exerts wide acting effects throughout the body. Despite its wide-ranging effects, suramin administration alone did not induce any change in lung function responses in naïve, acutely OA challenged or chronically OA challenged animals. Additionally, UTP administration alone induced no bronchodilator action in sensitised or chronically OA challenged animals. Furthermore, there was no bronchodilation to UTP after suramin administration in acutely OA challenged animals.

Thus, suramin revealed this bronchodilator response only in chronically OA challenged guinea pigs exposed to UTP. It has been demonstrated that suramin has the capacity to inhibit membrane-bound ecto-enzymes, which function to degrade ATP and UTP into their respective metabolites (Chen *et al* 1996). Therefore airway UTP levels following UTP exposure in the presence of suramin may be increased compared to levels following UTP exposure without suramin pretreatment. If this is true, it is plausible that the increase in sG_{aw} subsequent to UTP exposure in suramin pretreated, chronically OA challenged guinea pigs, may be due to a dose-dependent UTP-mediated bronchodilation. Furthermore, suramin is an antagonist at some pyrimidine-sensitive receptors but not others. For example, Charlton *et al* (1997) found that suramin has antagonist properties at the $P2Y_2$ receptors, but not at the human $P2Y_4$ receptor. It is possible that airway smooth muscle tone is maintained via different pyrimidine-sensitive receptors, each exerting opposite effects on airway smooth muscle. In the presence of suramin the inhibition of one receptor (which causes bronchoconstriction) and not the other (which causes bronchodilation), could result in a net bronchodilatory response. However, a single UTP exposure, 30mins subsequent to an i.p. injection of suramin, also did not induce any change in lung function in acutely OA challenged guinea pigs. There are two possible explanations for this. Firstly, the bronchodilator response following UTP and suramin administration in chronically OA challenged guinea pigs may be a result of airway remodelling and altered receptor expression during the chronic OA challenge. Secondly, it has previously been shown that chronically OA challenged guinea pigs reveal reduced baseline sG_{aw} values (Chapter 3, Fig. 3.11), suggesting increased baseline bronchoconstriction of the airways. Therefore, the bronchodilator response following UTP and suramin administration in chronically OA challenged guinea pigs may be due to an increased bronchoconstrictor airway tone in chronically OA challenged animals, which would favour revealing a bronchodilator action following bronchodilator administration.

4.6.4 DOSE-DEPENDENT GOBLET CELL-ASSOCIATED MUCIN SECRETION

In order to demonstrate potential dose-dependent UTP-induced lung responses, chronically OA challenged guinea pigs were exposed to a 10-fold increased concentration of UTP (10mM). However, both low dose and high dose UTP stimulated a comparable reduction in sG_{aw}, indicating that maximal response was achieved at 1mM UTP. In contrast however, whereas a nebulised exposure of low dose UTP (1mM) induced a 69.5% reduction in the mean % of AB/PAS-positive epithelial area, a 46.8% reduction was revealed following the high dose exposure (10mM), suggesting reduced mucin secretion following exposure to high dose UTP. Although unexpected, quantification of the area of AB/PAS-positive epithelial cells may not directly and accurately correlate with volumes of secreted mucin. Goblet cells containing different volumes of mucin may span the same area of bronchiolar epithelium depending on their 3-dimensional structure. It is therefore possible that similar volumes of goblet cell-associated mucin were secreted following both concentrations of UTP, despite disparity in the area of AB/PAS-positive bronchiolar epithelium.

4.6.5 TIME DEPENDENT GOBLET CELL-ASSOCIATED MUCIN SECRETION

Studies by Davis (1992) demonstrated that addition of luminal ATP stimulated an immediate release of goblet cell-associated mucin release and this was followed by a persistent release of mucin. These findings correspond with lung function responses following UTP exposure in chronically OA challenged guinea pigs, which reveal a gradual reduction in sG_{aw} reaching significance at 30mins. Additionally, histological analysis revealed that although there was no reduction in the mean % of AB/PAS-positive bronchiolar epithelial area at 0mins subsequent to UTP exposure, a significant reduction in the mean % of AB/PAS-positive bronchiolar epithelial area was revealed at 1hr subsequent to UTP exposure. This suggests that in our model, UTP-mediated goblet cell-associated mucin secretion was not instantaneous but was significant at some point between 0mins and 1hr subsequent to UTP exposure.

At 48hrs subsequent to UTP exposure, epithelial mucin accumulation was nearly restored to control levels, suggesting that the epithelium's capacity to store increased amounts of mucin persists for at least 48hrs. However, due to limited research it is unclear whether this effect is short term or long lasting.

4.6.6 ATP-MEDIATED GOBLET CELL-ASSOCIATED MUCIN SECRETION

In addition to UTP, the nucleotide ATP is also a full agonist at $P2Y_2$ receptors. ATP and UTP are equipotent in stimulating mucin release from hamster epithelial cells (Kim *et al* 1996) and ATP stimulates the degranulation of tracheal goblet cells in canine tracheal epithelium (Davis *et al* 1992). In contrast, work by Roger (2000) revealed that MUC5AC mucin secretion from goblet cells in human bronchi could be induced by ATP but not UTP. It was therefore of interest to investigate airway responses following ATP exposure in the guinea pig model of asthma. Although ATP revealed a reduction in sG_{aw}, similar to the pattern observed following UTP exposure, there was no significant difference compared to control. The lack of significance appeared to be due to large interindividual differences, possibly a result of the additional effects of ATP metabolites in the airways. Similarly, although ATP stimulated a reduction in the mean % of AB/PAS-positive bronchiolar epithelial area compared to control, it was not significantly different from control.

There is also evidence that UDP, the metabolite of UTP, may function as a weak agonist to regulate airway epithelial mucin secretion at $P2Y_2$ receptors (Chen *et al* 2001, Choi *et al* 2005). Like UTP, UDP stimulated a significant reduction in the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs, suggesting goblet cell-associated mucin secretion. However, in contrast to the response following UTP exposure, which persisted up to 1hr subsequent to UTP exposure, the reduction in lung function was maximal at 30mins and recovered at 45mins. These results support previous findings by Choi *et al* (2005) and Chen *et al* (2001) which state that UDP may act as a weak/partial agonist to stimulate airway mucin secretion.

4.6.7 EXPERIMENTAL LIMITATIONS

Both ATP and UTP are quickly broken down in the body by 11 plasma membrane-bound ecto-enzymes. ATP added to the surface of human nasal epithelial cells was metabolised by ectoenzymes with a half-life of 5mins (Morse *et al* 2001). The enzyme-catalysed inactivation of nucleotides is coupled with the formation of the breakdown nucleotides and nucleosides, which can also be pharmacologically active at alternative purinergic and pyriminergic receptors. It is also important to remember that the purity of commercially available nucleotides may be questionable and contamination with other nucleotides or breakdown products is likely. The catabolism of nucleotides and possible presence of degradation products complicates the analysis of biological responses to nucleotides and it is important to remember that responses observed following nucleotide or nucleoside administration might also be due to their respective metabolites or phosphorylation products (Brunschweiger and Muller 2006). Use of the more stable UTP analogues such as INS316 and INS315, developed by Inspire Pharmaceutics Inc. may reduce the accumulation of metabolites following exposure.
The use of suramin as a $P2Y_2$ receptor antagonist has limitations due to its non-selectivity. Suramin can act as an antagonist at additional P2 receptors and therefore, whilst it is likely, it cannot be conclusively stated that the effects of UTP in the airways of chronically OA challenged guinea pigs are mediated by $P2Y_2$ receptors. To specifically identify $P2Y_2$-mediated effects in the airways, a selective $P2Y_2$ receptor antagonist should be utilised.

4.6.8 ADDITIONAL EFFECTS OF NUCLEOTIDE TRIPHOSPHATES IN THE AIRWAYS

In addition to regulation of goblet cell mucin secretion in the airways, activation of epithelial cell $P2Y_2$ receptors by UTP can stimulate activation of chloride channels, resulting in Cl^- movement into the ASL and subsequent osmotic movement of water into the airway lumen (Tarran 2004). The subsequent increase in sol layer volume can enhance MCC by both the hydration and loosening of airway mucus and by aiding efficient ciliary beating. UTP can also stimulate an increase in CBF directly, probably due to increased intracellular calcium concentrations (Korngreen and Priel 1996). ATP

and UTP appear equipotent in stimulating ciliary beating, whilst UDP stimulates ciliary beating at half the effect of ATP and UTP (Morse *et al* 2001). Morse *et al* (2001) have revealed a short-term stimulation of CBF by UTP or ATP via $P2Y_2$ receptors and a long-term upregulation of CBF by ADO, mediated via A_{2b} receptors. Additionally, guinea pigs cough following UTP nebulised exposure and airway mucus clearance during cough may be increased subsequent to application of UTP to the airways (Noone *et al* 1999). It is therefore likely that mucus clearance is enhanced in the guinea pig subsequent to UTP and ATP nebulised exposures, which may lessen lung function responses subsequent to nucleotide exposures.

4.6.9 SUMMARY AND FURTHER WORK

In summary, it is proposed that the UTP-induced reduction in lung function is a result of P2 receptor, possibly $P2Y_2$, receptor-mediated goblet cell-associated mucin secretion by UTP, resulting in accumulation of mucus in the airways and subsequent reduction in lung function. However, despite demonstrating no UTP-induced bronchoconstriction in the normal guinea pig airway, there may be AHR to UTP after chronic OA challenge due to OA-mediated airway remodelling and potential altered receptor expression. Further investigation into the effect of the MARCKs-related peptide (Agrawal *et al* 2007) on lung function responses in chronically OA challenged guinea pigs may provide additional evidence of P2 receptor-mediated mucus secretion and resulting reductions in lung function. Furthermore, although UDP also appears to stimulate goblet cell mucin secretion, lung function responses were recovered more rapidly following UDP exposure compared to nebulised exposures to the triphosphates, ATP and UTP. This suggests that UDP may function as a weak/partial agonist at $P2Y_2$ receptors or once in the airways, may be quickly degraded by airway ecto-enzymes. This is the first study to show that lung function appears to be impaired by mucus secretion in an experimental animal model of chronic allergic asthma.

CHAPTER 5

The effect of nebulised 5'AMP exposure on goblet cell-associated mucin secretion and lung function changes in chronically OA challenged guinea pigs

5.1 INTRODUCTION

5.1.1 BIOSYNTHESIS AND METABOLISM OF ADENOSINE

Adenosine is a purine nucleoside, consisting of an adenosine molecule and a ribose group attached via a glycosidic linkage (for structure, see Fig. 5.1). It is an important biologically active molecule, present in all cells and ubiquitously distributed in the extracellular compartment.

The biosynthesis and metabolism of adenosine has been extensively studied and reviewed (Polosa 2002, Livingston *et al* 2004). Adenosine can be synthesised from amino acid precursors and ribose, but the majority of adenosine levels in the body are generated from the recycling of its phosphorylated derivatives AMP, ADP and ATP (Scaramuzzi *and* Baker 2003). During cellular energy metabolism, intracellular adenosine is liberated from the degradation of ATP, ADP and AMP. However, to prevent excessively high adenosine levels, adenosine can be reconverted into AMP via the action of adenosine kinase, and further phosphorylated into ADP and ATP. Alternatively, when energy demand is particularly high, under conditions of hypoxia, high levels of intracellular adenosine can be metabolised into inosine and hypoxanthine by adenosine deaminase (Livingston *et al* 2004).

The majority of the body's adenosine is produced from the dephosphorylation of extracellular adenosine 5 monophosphate (5'AMP) (for structure, see Fig. 5.2) by the membrane bound enzyme 5'nucleotidase. The diffusion of down its concentration gradient into the extracellular compartment, subsequent to its intracellular formation, provides the most important source of extracellular 5'AMP, whilst smaller amounts are produced by the degradation of extracellular ADP and ATP. Once formed, diffusion of extracellular adenosine into the cell occurs, via the energy independent nucleoside transporter (Polosa 2002). For a schematic diagram illustrating the intracellular and extracellular metabolism of adenosine, see Fig. 5.3.

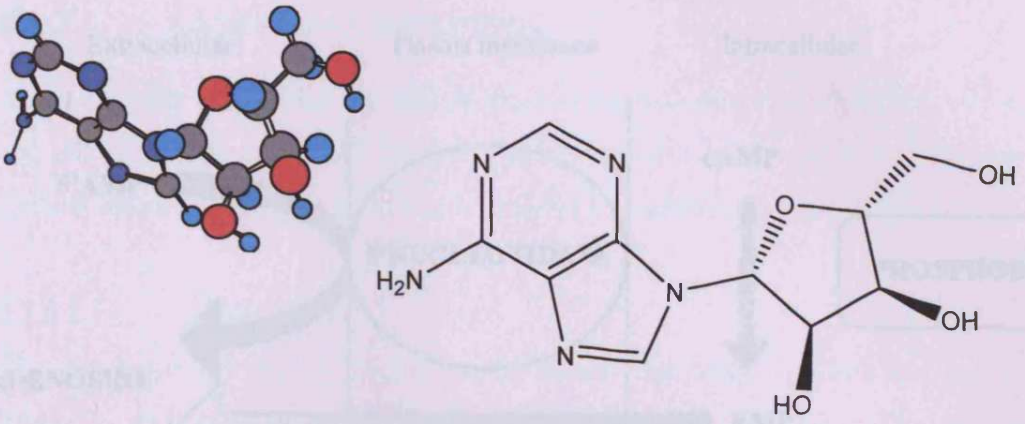


Figure 5.1. The chemical structure of adenosine

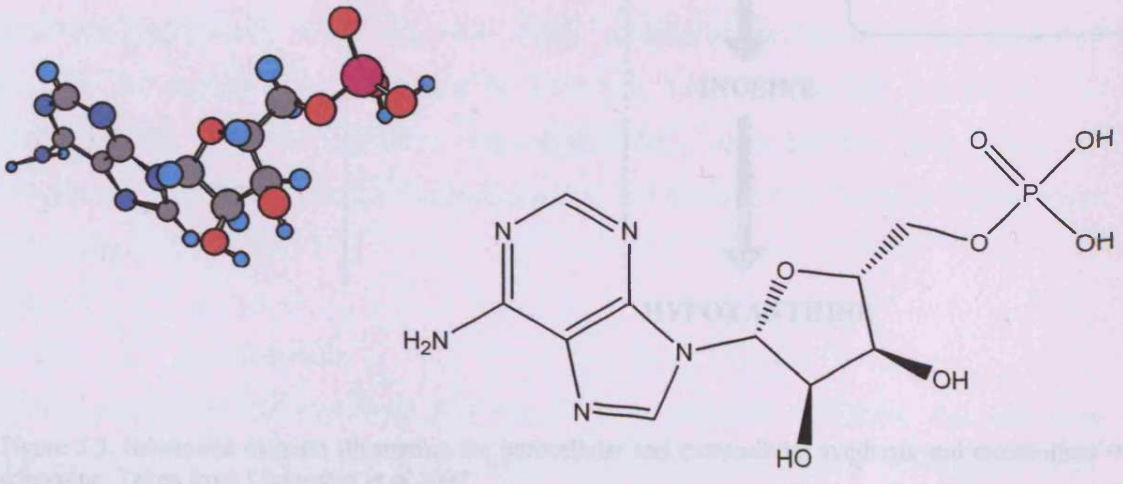


Figure 5.2. The chemical structure of adenosine 5'monophosphate

5.1.2 ACTIONS OF ADENOSINE

Adenosine is found in the extracellular compartment under normal conditions at an average concentration of 300nM. However, extracellular adenosine levels can increase significantly in times of stress or tissue damage (reviewed by MacLean *et al* 1998).

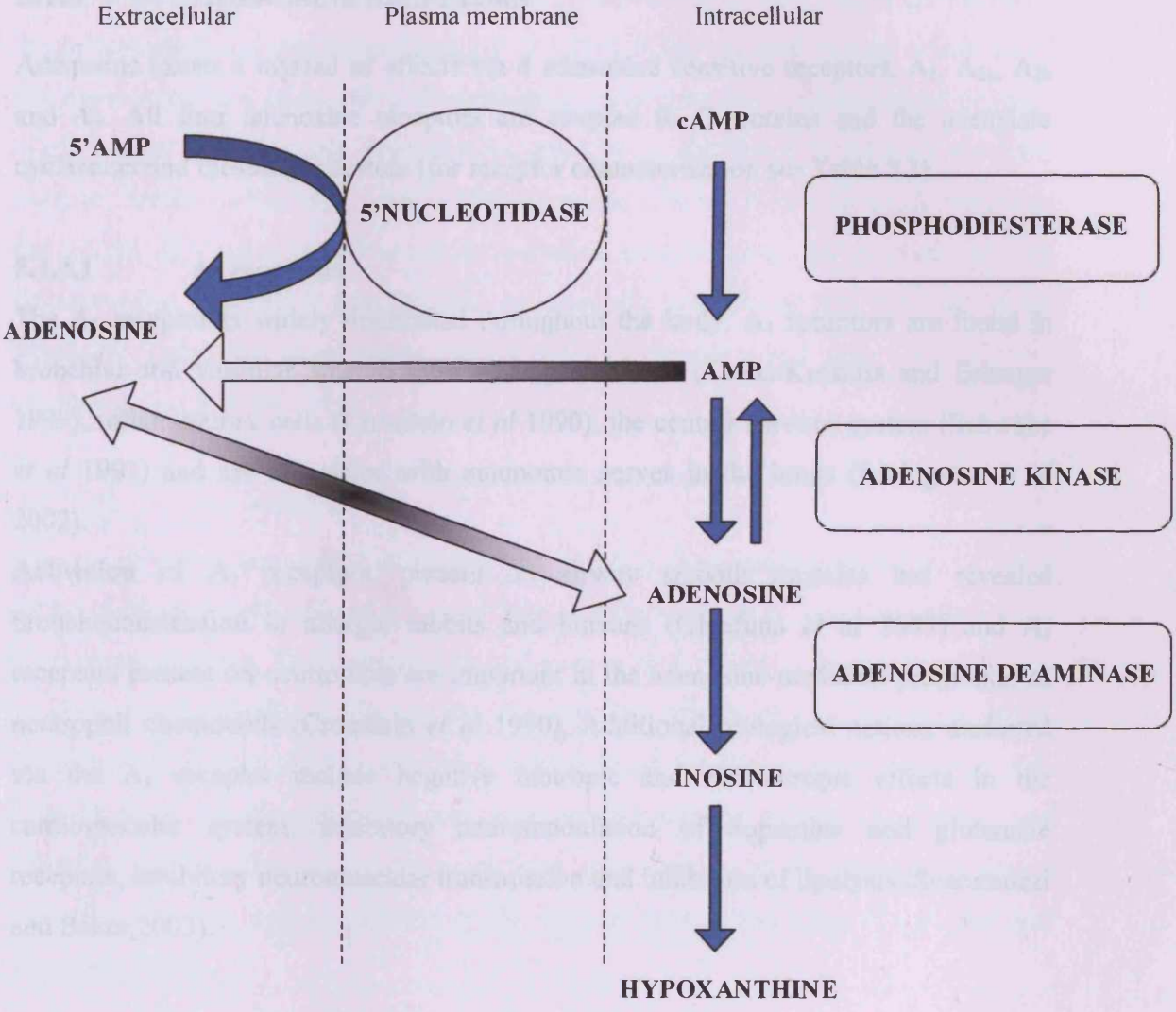


Figure 5.3. Schematic diagram illustrating the intracellular and extracellular synthesis and metabolism of adenosine. Taken from Livingston *et al* 2004

Adenosine produces a variety of effects throughout the body, including in the CNS, heart, vascular system, skeletal muscle and immune system. It can act as a hormone, an intracellular modulator and a neuromodulator (Scaramuzzi and Baker 2003).

5.1.3 ADENOSINE RECEPTORS

Adenosine exerts a myriad of effects via 4 adenosine sensitive receptors, A_1, A_{2a}, A_{2b} and A_3. All four adenosine receptors are coupled to G-proteins and the adenylate cyclase second messenger system (for receptor characterisation see Table 5.1).

5.1.3.1 A_1 receptors

The A_1 receptor is widely distributed throughout the body. A_1 receptors are found in bronchial and vascular smooth muscle, adipose tissue (Tatsis-Kotsidis and Erlanger 1999), inflammatory cells (Cronstein *et al* 1990), the central nervous system (Schwabe *et al* 1991) and are associated with autonomic nerves in the lungs (Livingston *et al* 2002).

Activation of A_1 receptors, present on airway smooth muscles has revealed bronchoconstriction in allergic rabbits and humans (Obiefuna *et al* 2005) and A_1 receptors present on neutrophils are important in the adenosine-mediated promotion of neutrophil chemotaxis (Cronstein *et al* 1990). Additional biological actions mediated via the A_1 receptor include negative inotropic and chronotropic effects in the cardiovascular system, inhibitory neuromodulation of dopamine and glutamate receptors, inhibitory neuromuscular transmission and inhibition of lipolysis (Scaramuzzi and Baker 2003).

5.1.3.2 A_2 receptors

The A_2 receptors are separated into two different receptor subtypes, A_{2a} and A_{2b}, depending on their high or low affinity for adenosine respectively (Livingston *et al* 2004)

5.1.3.2.1 A_{2a} receptor

The A_{2a} receptors are located in the vascular smooth muscle, endothelium, neutrophils (Sullivan *et al* 1999), platelets, mast cells, CNS (Sebastiao and Ribeiro 1996) and T lymphocyte inflammatory cells (T cells) (Livingston *et al* 2004). The A_{2a} receptors may play a role in the inflammatory response, inducing an anti-inflammatory action on a number of inflammatory cells (Khoa *et al* 2001). Activation of the A_{2a} receptors can induce suppression of histamine release from mast cells, bronchodilation (Hughes *et al*

1984), suppression of neutrophil chemotaxis (Cronstein *et al* 1992) and suppression of T cell activation and expansion (Huang *et al* 1997). In the vascular system, activation of the A_{2a} receptor can inhibit platelet aggregation and reduce heart rate and blood pressure (Ledent *et al* 1997). In the brain, the A_{2a} receptor is distributed in a highly localised manner, mainly in the basal ganglia (Sebastiao and Ribeiro 1996). There, it stimulates the release of several neurotransmitters including acetylcholine (ACh), glutamate and noradrenaline, acts as a neuromodulator, and interacts with several receptors such as the glutamate, calcitonin gene-related peptide (CGRP) receptors and dopamine receptors (Sebastiao and Ribeiro 1996).

5.1.3.2.2 A_{2b} receptors

The A_{2b} receptors are widely distributed and found in the brian (Sebastiao and Ribeiro 1996), epithelial cells (Murakami et al 2002), endothelial cells, smooth muscle cells (Lynge and Hellsten 2000), fibroblasts, and mast cells (Livingston *et al* 2004). The A_{2b} receptor is structurally similar to the A_{2a}, but the effects of A_{2b} activation differ considerably. Unlike the A_{2a} receptors, activation of the A_{2b} receptor on mast cells induces mast cell degranulation and subsequent mediator release (Feoktistov and Biaggiani 1995). The A_{2b} receptor has also been implicated in adenosine-induced intestinal secretion and vasodilation in the vascular system and lungs (Feoktistov and Biaggioni 1997).

5.1.3.3 A_3 receptor

The A_3 receptor is widely distributed (Livingston *et al* 2005). The mRNA has been identified in the testis, lung, kidneys, heart and CNS (Zhou *et al* 1992) and the receptor is found on mast cells (Keller 1997), eosinophils (Walker 1997) and neutrophils (Walker 1997). However, there are large species differences in the structure and distribution of the A_3 receptor. Despite the characterisation of mast cell A_3 receptors in several animals, they are not expressed in the human mast cell (Walker *et al* 1997). Activation of the A_3 receptor exerts several effects in the lungs. For example, activation of the A_3 receptor can stimulate mast cell degranulation and subsequent bronchoconstriction in animal models (Keller 1997). Additionally, A_3 receptors are located on eosinophils and neutrophils and may promote neutrophilic and eosinophilic

inflammation (Chen *et al* 2006). Finally, the A_3 receptor may also play a role in mucus production and secretion (Young 2006).

5.1.4 EFFECTS IN ASTHMA

Adenosine is formed constitutively in all cells under normal conditions. However, in conditions of high-energy demand, adenosine synthesis is upregulated and in hypoxia, resting extracellular adenosine levels can increase significantly (MacLean *et al* 1998). Adenosine is thought to play an important role in the asthmatic response. Adenosine can elicit inflammatory cell influx into the airways (Spruntulis and Broadley 2001). In the BALF of asthmatic patients, adenosine levels are raised (Driver *et al* 1993) and an increase in adenosine concentrations in the BALF of sensitised laboratory animals is observed following allergen challenge (Ali *et al* 1996). In human asthmatic airways, adenosine can elicit a significant bronchoconstriction response, but not in the normal lung. In the laboratory, inhaled adenosine or 5'AMP can elicit significant bronchoconstriction in sensitised guinea pigs (Smith and Johnson 2005), rats (Wyss *et al* 2005) and rabbits (Ali *et al* 1994). However, this airway response is not observed in non-sensitised animals (Thorne and Broadley 1992). There is much evidence suggesting that adenosine stimulates significant bronchoconstriction via mast cell degranulation. Adenosine-mediated mast cell degranulation and subsequent release of histamine, PGD_2 and tryptase, has been demonstrated *in vivo* and mast cell stabilisers such as cromolyn sodium and nedocromil sodium (Church and Holgate 1993) and H_1 receptor antagonists (Phillips *et al* 1989) can attenuate AMP-induced bronchoconstriction. In the rat, adenosine-mediated mast cell degranulation can be mediated via the A_{2b} and A_3 receptor (Fozard *et al* 1996). However clinically, adenosine-induced mast cell degranulation is likely mediated by A_{2b} receptors due to the lack of human mast cell A_3 receptors (Walker *et al* 1996).

Several cell types important in the pathogenesis of asthma express adenosine receptors including mast cells (Fozard *et al* 1996), T cells (Huang *et al* 1997), neutrophils (Cronstein *et al* 1990), macrophages (Sullivan *et al* 1999) and eosinophils, (Livingston *et al* 2004). Adenosine has both pro-inflammatory actions (via A_1, A_{2b} and A_3) and anti-inflammatory actions (via A_{2a}). Activation of the A_{2a} receptor induces suppression of inflammatory cell chemotaxis, activation and proliferation, as discussed earlier. The

anti-inflammatory effect of adenosine, mediated by the high affinity A_{2a} receptor, appears to predominate in normal conditions when adenosine levels are low. However, during inflammation, adenosine levels are increased and the pro-inflammatory effect of adenosine, mediated by the low affinity A_{2b} receptors, dominates (Polosa *et al* 2002). Adenosine also appears to induce mucus production, in cooperation with inflammatory cytokines (McNamara *et al* 2004) and stimulates mucus secretion from airway secretory cells via activation of the A_3 receptor (Young 2006).

5.1.5 AGONISTS AND ANTAGONISTS

A number of adenosine receptor agonists and antagonists are available (see Table 5.1)

Adenosine receptor	**A_1**	**A_{2a}**	**A_{2b}**	**A_3**
G-protein	G_i (modulates cAMP)	G_s (increases cAMP)	G_s (increases cAMP)	G_i (cAMP modulation)
Intracellular signalling	· inhibits adenylate cyclase · inhibits Ca^{2+} conductance · increases K^+ conductance	· stimulates adenylate cyclase · stimulates phospholipase activity	· stimulates adenylate cyclase · increases Ca^{2+}conductance	· inhibits adenylate cyclase · stimulates phospholipase activity · increases Ca^{2+}conductance
Effects	· inhibitory neuromodulation of dopamine and glutamate receptors · negative inotropic and chronotropic effects in the cardiovascular system · inhibitory neuromuscular transmission · inhibit lipolysis, stimulate glucose	· vasodilation · inhibits platelet aggregation · stimulatory neuromodulation of dopamine and glutamate receptors · stimulatory neuromuscular transmission · involved in carbohydrate metabolism in Type I muscle	· involved in carbohydrate metabolism in Type I muscle fibres	

	uptake and induce insulin resistance	fibres		
Agonists	CPA GR79236	CGS21680 CV1808 DPMA HE-NECA	Metrifudil NECA	NECA IB-MECA
Antagonists	8PT DPCPX N-0840 CPT	8PT ZM241385 SCH-58261 ZM241,385	8PT MRS1706 MRE 2029-F20	MRS1220 MRS1191 MRS1292 MRS1523

8PT: 1,3-dimethyl-8-phenyl-xanthine. CGS21680: 2-p-(2-Carboxethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine. CPA: (N-(6)-cyclo-pentyl-adenosine). CPT: 8-Cyclopentyl-1,3-dimethylxanthine. CV1808: 2-Phenylaminoadenosine. DPCPX: 1,3-dipropyl-8-cyclo-pentyl-xanthine. DPMA: N^6-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine. G_i: Inhibitory G protein. GR79236: N-[(1S, trans)-2-hydroxyxyxlopentyl]adenosine. G_s: stimulatory G protein. HE-NECA: 2-Hexynyl-adenosine-5'-N-ethyluronamide. IB-MECA: N6-(3-Iodobenzyl)-9-[5-(methylcarbamoyl)-β-D-ribofuranosyl]adenine. MRE 2029-F20: N-Benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]-acetamide. MRS1191: 3-Ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethylnyl-1,4-(±)-dihydro-pyrimidine-3,5-dicarboxylate. MRS1220: 9-chloro-2-(2-furyl)-5-[(phenyl acetyl)amino][1,2,4]triazol[1,5-c] quinazoline. MRS1292: (2R, 3R, 4S, 5S)-2-[N6-3-Iodobenzyl)adenos-9'-yl]-7-aza-1-oxo-spirol[4.4]-nonan-4,5-diol. MRS1523: 2,3-Diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate. MRS1706: N-4 (acetyl phenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-diox-1,3-dipropyl-1H-purin-8-yl) phenoxy]acetamide. N-0840: N6-Cyclopentyl-9-methyladenine. NECA: N-Ethylcarboxamidoadenosine. SCH-58261: 5-Amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo(4,3-e)-1,2,4-triazolo(1,%-c)pyrimidine. ZM241,385: 4-[2-(7-Amino-2-(2-furyl)[1,2,4-triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl phenol. ZM241385: 4-(2-[7amino-2-(2 furyl[1,2,4] -triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl) phenol.

Table 5.1. Adenosine receptors pharmacology (Adapted form Scaramuzzi and Baker 2003, SigmaAldrich 2007)

5.2 AIMS AND OBJECTIVES

HYPOTHESIS. *In a chronically OA challenged guinea pig, a nebulised exposure of the secretagogue, adenosine, induces goblet cell degranulation and mucus secretion, which leads to airway mucus accumulation and ultimately a reduction in lung function.*

5.2.1 AIM

The aim of this chapter was to utilise plethysmography and histological methods to analyse the effect of nebulised 5'AMP exposures on lung function responses and goblet cell-associated mucin secretion.

5.2.2 OBJECTIVES

- To identify airway function responses in sensitised guinea pigs following a single nebulised exposure of 5'AMP.
- To identify lung function responses following a single nebulised exposure of 5'AMP in acutely OA challenged guinea pigs
- To identify lung function responses following a single nebulised exposure of 5'AMP in chronically OA challenged guinea pigs
- To reveal the effects of a single nebulised exposure of 5'AMP on the mean % of AB/PAS-positive bronchiolar epithelial area.

5.3 METHODS

Groups of 6 male Dunkin-Hartley guinea pigs (supplied by Harlan, UK) weighing between 200-250g were used for all protocols.

5.3.1 SENSITISATION

Animals were sensitised on days 1 and 5 with an i.p, bilateral injection of a suspension containing 100 μg of OA and 100 mg aluminium hydroxide.

5.3.2 NEBULISED OVALBUMIN EXPOSURES

14 days subsequent to the sensitisation period (day 15), guinea pigs were challenged with either an acute OA challenge or chronic OA challenge. For all OA challenges, a Wright nebuliser was used to supply air at a pressure of 20p.s.i. and at a rate of 0.3ml/min into a sealed stainless steel exposure chamber (40cm diameter, 15cm height). If any animal appeared in distress, the animal was removed from the exposure chamber and the challenge considered complete.

5.3.2.1 Acute OA exposures

14 days following sensitisation (day 15), animals were exposed to a nebulised solution of low dose OA (0.01% for 1hr).

5.3.2.2 Chronic OA exposures

14 days following sensitisation (day 15), animals were exposed to a single nebulised solution of low dose OA (0.01% for 1hr). Animals were subsequently exposed to a nebulised solution of high dose OA (0.1% for 1hr) on days 17, 19, 21, 23, 25, 27 and 29. Mepyramine (30mg/kg) was administered by bilateral, i.p. injection 30mins prior to OA challenge on days 17, 19, 21, 23, 25 and 27.

5.3.3 NEBULISED EXPOSURES TO 5'AMP

Sensitised guinea pigs not challenged with OA (day 15), acutely challenged to OA (day 16, 24hrs subsequent to acute OA challenge) or chronically challenged to OA (day 30,

24hrs subsequent to the last OA challenge) were exposed to a nebulised solution of 5'AMP (3mM for 1mins) or vehicle (1min). For all exposures, a Wright nebuliser was used to supply air at a pressure of 20p.s.i. and at a rate of 0.3ml/min into a sealed perspex chamber (15x 15x 32cm). Lung function responses were measured immediately prior to 5'AMP exposure and at 0, 5, 10, 15mins and every 15mins up to 1hr subsequent to 5'AMP exposure.

5.3.4 NEBULISED EXPOSURES TO UTP

UTP was used in this chapter to allow comparison of lung responses following 5'AMP exposure to lung responses following UTP exposures. 22hrs 45mins subsequent to chronic OA challenge (day 30), guinea pigs were exposed to a nebulised solution of UTP (10mM for 15mins) as described in Chapter 4. Lung function responses were measured immediately prior to UTP exposure and at 0, 5, 10, 15mins and every 15mins up to 1hr subsequent to UTP exposure.

5.3.5 LUNG FUNCTION MEASUREMENTS

Whole body plethysmography was used to measure specific airway conductance (sG_{aw}) as previously described (Chapter 2).

5.3.6 HISTOLOGICAL ANALYSIS OF GUINEA PIG LUNGS

Immediately following lavage, lungs were removed from the thoracic cavity and fixed with formaldehyde. 3-5mm tangentially sliced portions of lung were processed into wax blocks, sectioned (3μm) using a Leica microtome and fixed onto glass slides. Slides were stained with AB/PAS and Mayers haemolum and each bronchiole analysed to give the % of AB/PAS-positive area of the bronchiolar epithelium. This was calculated for each bronchiole and mean values calculated. Detailed methodology is described in Chapter 2.

5.4 RESULTS

5.4.1 THE EFFECT OF 5'AMP CHALLENGE ON LUNG FUNCTION RESPONSES IN SENSITISED GUINEA PIGS

Fig. 5.4 represents the mean time course for changes in sG_{aw} following exposure to nebulised 5'AMP (3mM for 1min) or vehicle (1min) in sensitised guinea pigs. A significant early phase reduction in sG_{aw}, compared to vehicle exposure was revealed (-25.20 ± 5.67 %) immediately following 5'AMP exposure. The early phase reduction in lung function was recovered at 1hr and was followed by a late phase reduction in sG_{aw} (-15.16 ± 7.52 %) 7hrs subsequent to 5'AMP challenge

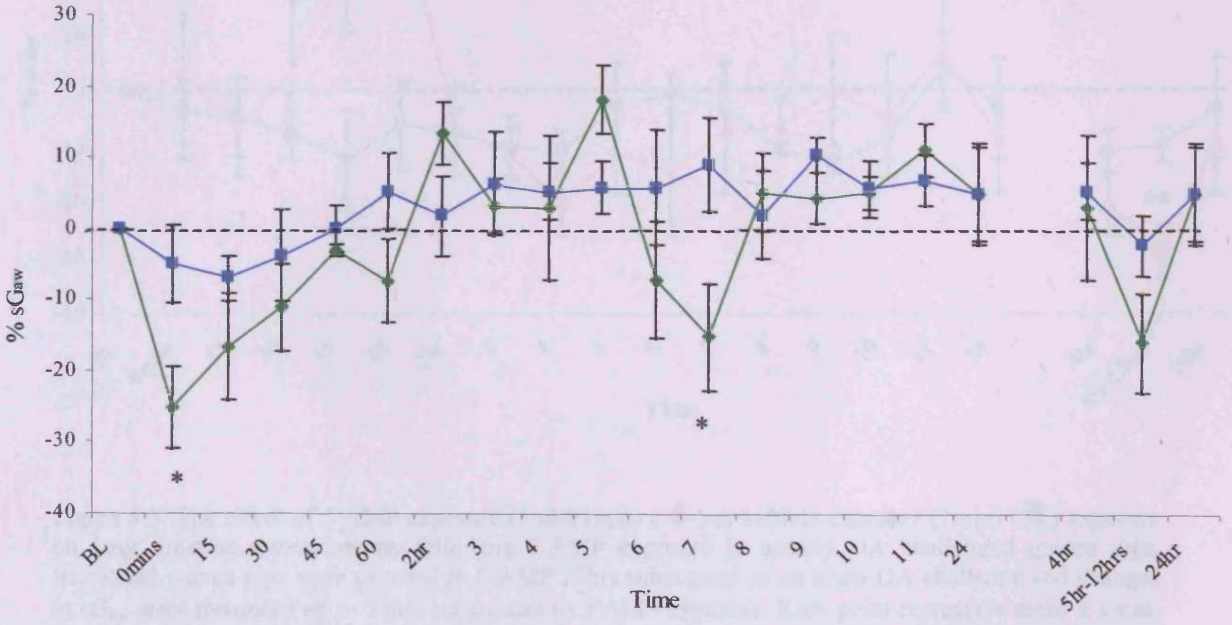


Figure 5.4. The effect of 5'AMP (3mM/1min) (◆) or vehicle (1min) (■) exposure on lung function measurements, up to 24 hours subsequent to 5'AMP challenge, in sensitised guinea pigs. Each point represents mean ± s.e.m. % change in sG_{aw} compared to baseline. The mean fall in sG_{aw} between 5 and 12hrs is also shown. n=6. * ($p<0.05$) significantly different from vehicle.

5.4.2 THE EFFECT OF 5'AMP CHALLENGE ON LUNG FUNCTION RESPONSES IN ACUTELY OA CHALLENGED GUINEA PIGS

Fig. 5.5 represents the mean time course for changes in sG_{aw} following exposure to nebulised 5'AMP (3mM for 1min) or vehicle (1min) exposure, 24hrs subsequent to an acute OA challenge in sensitised guinea pigs. 30mins subsequent to 5'AMP exposure, a significant early phase increase in sG_{aw} was revealed (+33.95 ± 10.08 %) and recovered at 2hrs. A significant late phase reduction in lung function was revealed following 5'AMP challenge (-28.76 ± 4.81%).

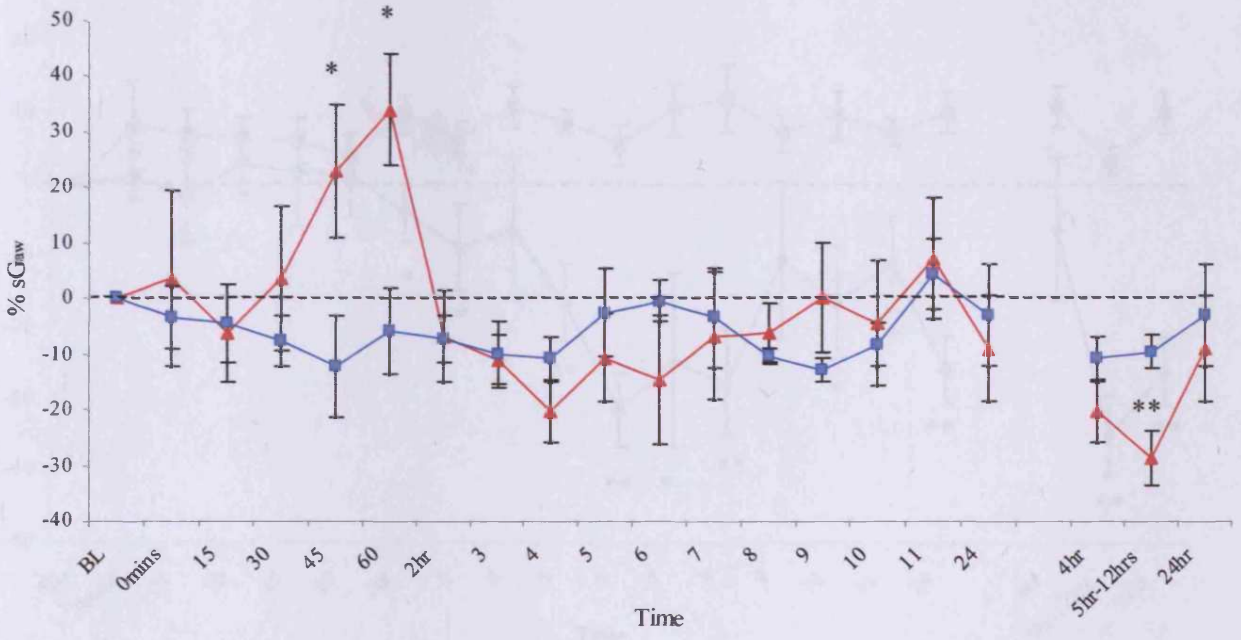


Figure 5.5. The effect of 5'AMP exposure (3mM/1min) (▲) or vehicle exposure (1min) (■) exposure on lung function measurements following 5'AMP exposure in acutely OA challenged guinea pigs. Sensitised guinea pigs were exposed to 5'AMP 24hrs subsequent to an acute OA challenge and changes in sG_{aw} were measured up to 24hrs subsequent to 5'AMP exposure. Each point represents mean ± s.e.m. % change in sG_{aw} compared to baseline. The mean fall in sG_{aw} between 5 and 12hrs is also shown. n=6. *($p<0.05$) **($p<0.01$) significantly different from vehicle

5.4.3 THE EFFECT OF 5'AMP CHALLENGE ON LUNG FUNCTION RESPONSES IN CHRONICALLY OA CHALLENGED GUINEA PIGS

Fig. 5.6 represents the mean time course for changes in sG_{aw} following exposure to nebulised 5'AMP exposure (3mM for 1min), 24hrs subsequent to the final OA challenge in chronically OA challenged guinea pigs. No significant change from baseline sG_{aw} was observed up to 1hr subsequent to 5'AMP exposure. However, nebulised 5'AMP induced a significant, prolonged late phase reduction in sG_{aw}, beginning at 2hrs and continuous until 24hrs.

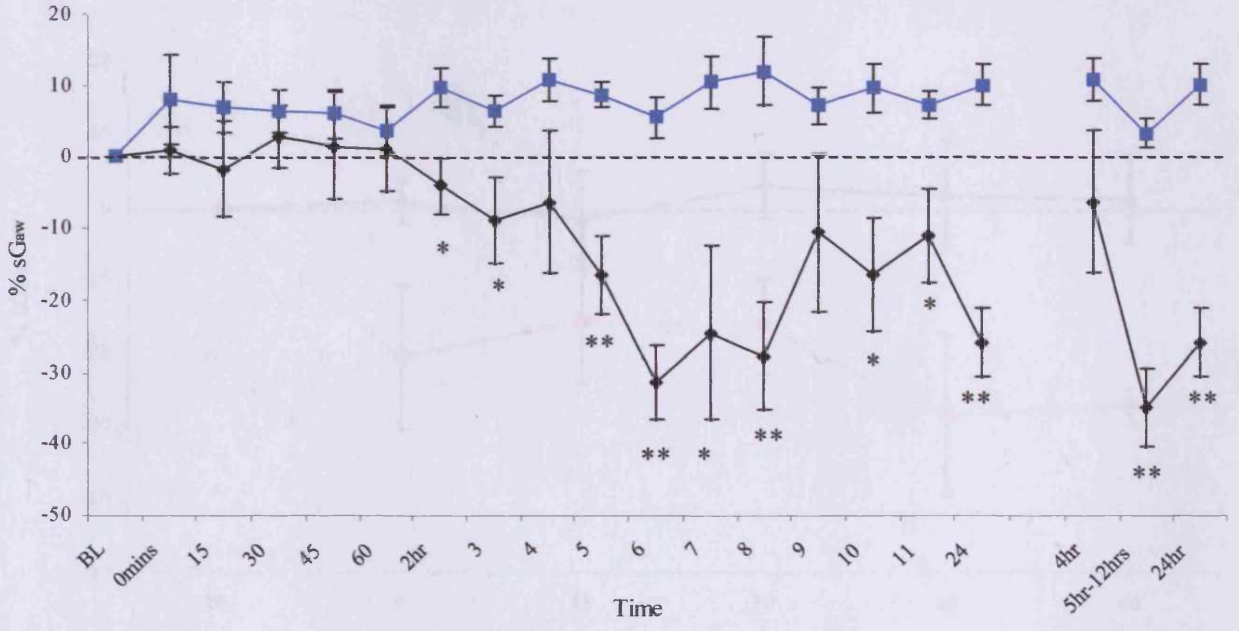


Figure 5.6. The effect of 5'AMP exposure (3mM/1min) (◆) or vehicle exposure (1min) (■) on lung function measurements, up to 24 hours after 5'AMP challenge, in chronically OA challenged guinea pigs. Sensitised guinea pigs were exposed to 5'AMP 24hrs subsequent to a chronic OA challenge and changes in sG_{aw} were measured up to 24hrs subsequent to 5'AMP exposure. Each point represents mean ± s.e.m. % change in sG_{aw} compared to baseline. The mean fall in sG_{aw} between 5 and 12hrs is also shown. n=6. * ($p<0.05$) ** ($p<0.01$) statistically different from vehicle.

5.4.4 COMPARISON OF NEBULISED 5'AMP AND UTP EXPOSURE ON LUNG FUNCTION RESPONSES IN CHRONICALLY OA CHALLENGED GUINEA PIGS

Fig. 5.7 represents the mean time course for changes in sG_{aw} following exposure to nebulised 5'AMP exposure (3mM for 1min) or UTP exposure (1mM for 15mins), 24hrs following the final OA challenge in chronically OA challenged guinea pigs. A single nebulised UTP exposure induces a prolonged reduction in lung function up to 1hr subsequent to exposure. No significant change from baseline sG_{aw} was observed up to 1hr subsequent to 5'AMP exposure.

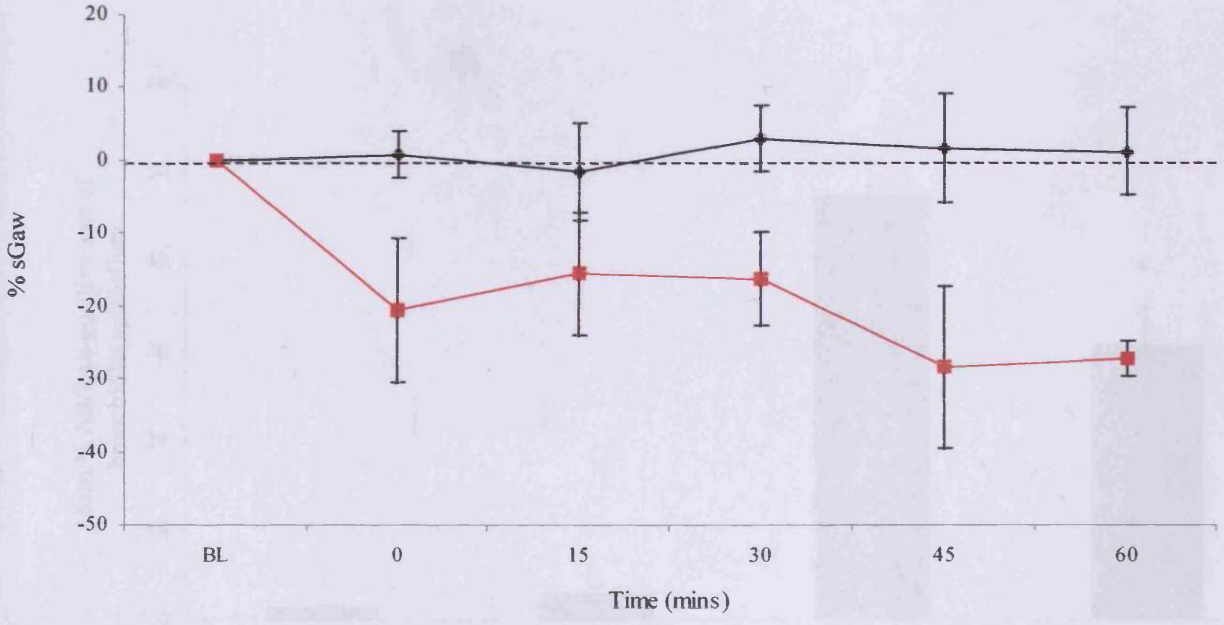


Figure 5.7. The effect of 5'AMP exposure (3mM/1min) (◆) or UTP exposure (10mM/15mins) (■) on lung function measurements, in chronically OA challenged guinea pigs. Sensitised guinea pigs were exposed to 5'AMP or UTP on day 30 of a chronic OA challenge and changes in sG_{aw} were measured up to 1hr subsequent to 5'AMP and UTP exposure. n=6. Each point represents mean ± s.e.m. % change in sG_{aw} compared to baseline.

5.4.5 EFFECT OF NEBULISED 5'AMP OR UTP EXPOSURES ON THE MEAN % OF AB/PAS-POSITIVE BRONCHIOLAR EPITHELIAL AREA IN ACUTELY AND CHRONICALLY OA CHALLENGED GUINEA PIGS

The mean % of AB/PAS-positive bronchiolar epithelial area was not significantly different in acutely OA challenged guinea pigs exposed to 5'AMP (3mM for 1min) compared to acutely OA challenged guinea pigs exposed to vehicle. However, the mean % of AB/PAS-positive bronchiolar epithelial area was significantly different in chronically OA challenged guinea pigs exposed to 5'AMP (3mM for 1mins) compared to chronically OA challenged guinea pigs exposed to vehicle (Fig. 5.8).

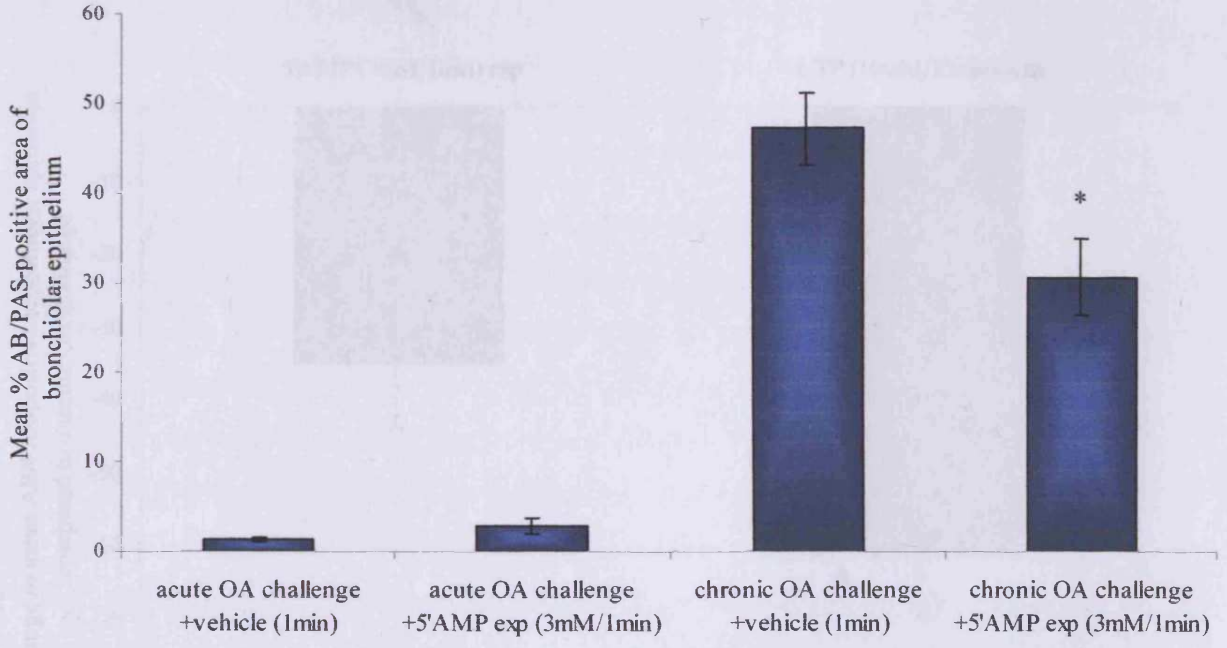


Figure 5.8. The mean % of AB/PAS bronchiolar epithelial area in 1) acutely OA challenged guinea pigs exposed to a single vehicle exposure (1min). 2) acutely OA challenged guinea pigs exposed to a single nebulised 5'AMP exposure. (3mM/1min). 3) chronically OA challenged guinea pigs exposed to a single vehicle exposure (1min). 4) chronically OA challenged guinea pigs exposed to a single nebulised 5'AMP exposure. Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of guinea pig left lung. n=5. * ($p<0.05$) significantly different to vehicle.

5.4.6 COMPARISON OF THE EFFECT OF UTP AND 5'AMP NEBULISED EXPOSURES ON THE % OF AB/PAS-POSITIVE BRONCHIOLAR EPITHELIUM IN CHRONICALLY OA CHALLENGED GUINEA PIGS

Both nebulised 5'AMP and UTP exposures induced a reduction in the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs. The % change in mean AB/PAS-positive bronchiolar epithelial area following 5'AMP or UTP exposure compared to chronically OA challenged guinea pigs exposed to vehicle was calculated. Nebulised UTP exposure induced a greater reduction (-61.0 ± 8.8%) in the mean % of AB/PAS-positive epithelial area, compared to nebulised 5'AMP exposure (-35.1 ± 9.0%) (Fig. 5.9).

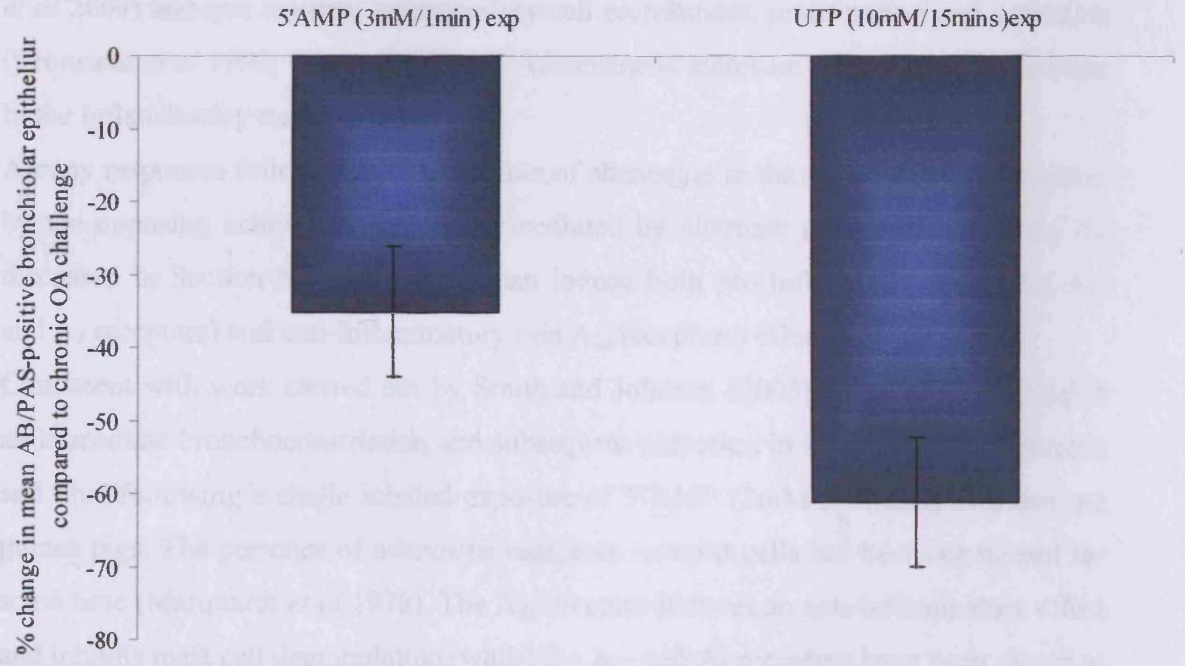


Figure 5.9. The % change in mean AB/PAS-positive bronchiolar epithelial area following either a single nebulised 5'AMP exposure (3mM/1min) or a single nebulised UTP exposure (1mM/15mins) in chronically OA challenged guinea pigs, compared to chronically OA challenged guinea pigs exposed to vehicle

5.5 DISCUSSION

In a guinea pig model of acute asthma (discussed in Chapter 3), the early phase asthmatic response following a single exposure of inhaled OA is thought to be mainly mediated via mast cell degranulation, subsequent histamine release and H_1-mediated bronchoconstriction. The late phase asthmatic response is thought to be a result of a complex inflammatory cascade, mediated by numerous inflammatory cell types and inflammatory mediators. Adenosine can also stimulate early and late bronchoconstriction responses in sensitised animals (Smith and Johnson 2005) and it is widely accepted that the adenosine-induced EAR in sensitised animals is also a result of mast cell degranulation and subsequent histamine release (Church and Holgate 1993). In addition, adenosine receptors are present on several cells important in the immune response, such as eosinophils, macrophages, neutrophils and lymphocytes (Livingston *et al* 2004) and can regulate inflammatory cell recruitment, proliferation and activation (Cronstein *et al* 1990, Chen *et al* 2006). Adenosine is therefore a likely potent mediator in the inflammatory response.

Airway responses following administration of adenosine in the airways are complicated by the opposing actions of adenosine, mediated by alternate adenosine receptors. As discussed in Section 5.1.3, adenosine can induce both pro-inflammatory (via A_1, A_{2b} and A_3 receptors) and anti-inflammatory (via A_{2a} receptors) effects.

Consistent with work carried out by Smith and Johnson (2005), these studies revealed an immediate bronchoconstriction and subsequent reduction in lung function between 6 and 8hrs following a single inhaled exposure of 5'AMP (3mM for 1min) in sensitised guinea pigs. The presence of adenosine receptors on mast cells has been considered for some time (Marquardt *et al* 1978). The A_{2a} receptor induces an anti-inflammatory effect and inhibits mast cell degranulation, whilst the A_{2b} and A_3 receptors have been shown to induce mast cell degranulation and subsequent histamine release. Additionally, activation of A_1 receptors, present on airway smooth muscles may stimulate bronchoconstriction directly (Obiefuna *et al* 2005) whilst simultaneous activation of A_{2a} receptors on airway smooth muscle may directly stimulate bronchodilation. It is therefore likely that the early phase response following 5'AMP exposure is a result of mast cell degranulation via the A_{2b} and A_3 receptors and possibly direct adenosine-

induced bronchoconstriction via the A_1 receptor. The bronchodilatory effects of the A_{2a} receptor appear to be insignificant during the early phase adenosine-mediated response in sensitised guinea pigs.

In acutely OA challenged guinea pigs, the 5'AMP-induced early phase bronchoconstriction is converted to a bronchodilatory response. This is likely to be a result of a reduction in the A_{2b} and A_3-mediated bronchoconstriction. This may have been caused by depletion of the airway mast cell population at 24hrs following acute OA challenge, resulting in reduced adenosine-mediated mast cell histamine release and subsequent H_1-mediated bronchconstriction. However, despite loss of early phase bronchoconstriction, acutely OA challenged guinea pigs reveal a premature and prolonged late phase reduction in sG_{aw}. If loss of the early phase bronchoconstriction is due to depletion of the airways activated mast cell population, then the late onset bronchoconstriction must be independent of mast cells. The late asthmatic response is thought to be due to an inflammatory cascade resulting in the recruitment and activation of various inflammatory cells and production of multiple inflammatory mediators. Adenosine receptors are present on the surface of several inflammatory cells such as neutrophils, eosinophils, macrophages, and adenosine has been shown to stimulate chemotaxis, proliferation and activation of inflammatory cells. Therefore, it is possible that 1) adenosine may activate previously recruited inflammatory cells (subsequent to acute OA challenge) and/or 2) adenosine may directly stimulate a late onset inflammatory response via the activation and recruitment of inflammatory cells.

In chronically OA challenged guinea pigs, a single exposure to inhaled 5'AMP, 24hrs subsequent to the chronic OA challenge did not induce an early phase bronchoconstriction or bronchodilation, but did stimulate a prolonged late inflammatory response between 2 and 24hrs. This differs significantly to responses observed in acutely OA challenged guinea pigs and is likely to be due to airway remodelling and possible alterations in airway receptor expression as a result of chronic allergen challenge. Alternatively, it may be due to mast cell depletion following previous OA challenges. Additionally, inflammatory cell recruitment in chronically OA challenged guinea pigs is significantly increased 24hrs subsequent to chronic OA challenge, compared to acutely OA challenged and sensitised guinea pigs (Chapter 3). Therefore, adenosine-induced activation and proliferation of previously recruited airway

inflammatory cells may stimulate the prolonged late inflammatory response observed following adenosine exposure in chronically OA challenged guinea pigs.

In the previous chapter, UTP was shown to induce a reduction in the mean % of AB/PAS-positive bronchiolar epithelial area, which was associated with a gradual reduction in lung function up to 1hr subsequent to UTP exposure (Fig. 4.2 and 4.7). It was proposed that the UTP-mediated reduction in lung function was due to goblet cell mucin secretion and accumulation within the airways. Whilst less potent than its triphosphate and diphosphate counterparts (Chen *et al* 2001), adenosine can also stimulate mucus secretion from airway epithelial secretory cells via activation of the A_3 receptor (Young 2006). It was therefore of interest to investigate the effects of nebulised adenosine on the AB/PAS-positive area of the bronchiolar epithelium and lung function responses in chronically OA challenged guinea pigs. A single nebulised exposure of 5'AMP induced a significant reduction in the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs, suggesting goblet cell-associated mucin secretion. However, the 5'AMP-induced reduction in epithelial-stored mucin was significantly less than that observed following UTP exposure, indicating that 5'AMP/adenosine is a less potent agonist than UTP in stimulating goblet cell-associated mucin secretion. Additionally, nebulised adenosine induced no change in sG_{aw} from baseline sG_{aw} values in chronically OA challenged guinea pigs up to 1hr following 5'AMP exposure. I suggest two possible reasons for this. Firstly, adenosine induces 50% less goblet cell-associated mucin secretion compared to UTP. This volume may not be sufficient to induce a reduction in lung function. Secondly, the possible reduction in lung function responses following adenosine exposure may be hidden by simultaneous A_{2a} -mediated bronchodilation.

In these studies, 5'AMP was utilised as a source and precursor of adenosine. 5'AMP is rapidly hydrolysed to adenosine in the airways and use of 5'AMP, rather than adenosine, was preferred in these studies due to its greater water solubility. However, it is important to remember that airway responses subsequent to 5'AMP exposure may be due to the effects of 5'AMP as well as adenosine.

In conclusion, an inhaled exposure of 5'AMP has been shown to induce goblet cell-associated mucin secretion, but that adenosine is a far less potent agonist at stimulating goblet cell-associated mucin secretion compared to UTP. However, lung responses to

adenosine are complicated due to the multiple biological actions of adenosine and its receptor subtypes. Further investigation using adenosine antagonists and mast cell stabilisers would help to distinguish which receptors and whether mast cell degranulation mediated these responses.

CHAPTER 6

The effect of histamine exposure on goblet cell-associated mucin secretion and lung function changes in chronically OA challenged guinea pigs

6.1 INTRODUCTION

6.1.1 SYNTHESIS AND METABOLISM OF HISTAMINE

The function of histamine, as well as its synthesis and metabolism has been reviewed in Akdis and Blaser (2003). The basic amine histamine (2-[4-imodazole]-ethylamine) functions as an autocoid, a potent physiological mediator in the gut and is a vital component of the immune response (see Fig. 6.1 for chemical structure). The synthesis of histamine from histidine is catalysed by the enzyme histidine decarboxylase in the tuberomammillary nucleus. Subsequent to its synthesis, histamine is stored throughout the body in several cell types including mast cells, basophils and enterochromaffin cells (Leurs and Timmerman 2000), and is found at particularly high concentrations in the skin, lungs and gastrointestinal tract. In mast cells, histamine is stored in intracellular cytoplasmic granules with large amounts of macro-heparin, anionic proteoglycans and chondroitin sulphate. Release of histamine via exocytosis of mast cell intracellular granules can be stimulated by various stimuli such as direct injury, activated complement, activation of mast cell-surface adenosine receptors or interaction of mast cell-fixed IgE with antigen. Once released from the cell, histamine is rapidly deactivated via two pathways: methylation and oxidative deamination. Methylation is the major pathway involved in the metabolism of histamine. Histamine is metabolised into N-methylhistamine and subsequently methylimidazole acetic acid by the two enzymes, N-methyl-transferase and monoamine oxidase respectively. Oxidative deamination is catalysed by the enzyme diamine oxidase and results in the formation of imidazole acetic acid (Akdis and Blaser 2003).

6.1.2 HISTAMINE RECEPTORS

Histamine has been recognised as an important physiological mediator for over 100yrs, but it was in 1966 that Ash and Schild first categorised the histamine receptors into H_1 and H_2 (Ash and Schild 1997). Since then, the search for inhibitors of histamine-induced responses has led to the discovery of the H_3 receptor in the 1980s by Arrang *et al* (1987) and most recently the H_4 receptor (Oda and Matsumoto 2001). All four

histamine receptors, H_1, H_2, H_3 and H_4, are G protein receptors, coupled to a range of second messenger systems.

The H_1 receptor belongs to the $G_{\alpha q}/G_{\alpha 11}$ family and is coupled with the inositol phosphate second messenger system, resulting in the formation of DAG and IP_3 and an increase in the concentration of intracellular calcium. Activation of the receptor has additionally revealed NO production, accumulation of cAMP, cGMP, phospholipase A_2, phospholipase D (Akdis and Blaser 2003) and activation of the transcription factor NF-κB (Aoki et al 1998). The H_2 receptor belongs to the $G\alpha_s$ family and is coupled to both adenylate cyclase, resulting in increases in intracellular cAMP and the inositol phosphate second messenger system (Wang *et al* 1996). It may also activate c-Fos, c-Jun, protein kinase C and p70S6 kinase (Akdis and Blaser 2003). The H_3 receptor is found at presynaptic membranes and mediates the release of several neurotransmitters. Activation of the H_3 receptor results in increases in intracellular calcium, inhibition of cAMP and activation of the mitogen-activated protein kinase pathway. There is limited information regarding the H_4 receptor. It belongs to the $G_{i/o}$ family and inhibits cAMP formation (Akdis and Blaser 2003).

6.1.3 THE BIOLOGICAL ACTIONS OF HISTAMINE

Histamine was one of the first mediators implicated in asthma. It is released from mast cells following mast cell degranulation (stimulated by interaction of antigen with mast cell-fixed IgE) and is thought to be the main mediator involved in the early asthmatic response. It subsequently binds to histamine-specific receptors to produce multiple well-characterised effects. These effects contribute to many of the clinical symptoms of asthma, and are mainly mediated via the H_1 receptor.

H_1 receptors are expressed on airway smooth muscle, endothelial cells, neurons, dendritic cells, mast cells, macrophages and lymphocytes (Togias 2003). Activation of H_1 receptors stimulates a numerous biological actions including bronchoconstriction, microvascular leakage, vasodilation, vasoconstriction, sensory nerve stimulation (Barnes *et al* 1991) and activation of cells of the immune system, inducing a proinflammatory effect (Bryce *et al* 2006). The H_1 receptor may also indirectly contribute to the symptoms of asthma by the induction of goblet cell mucus secretion following H_1 receptor activation on cholinergic nerve terminals and subsequent ACh

release. In animal models of asthma, antagonism of the H_1 receptor has revealed diminished allergic responses including early phase bronchoconstriction, lung Th2 cytokine levels, airway inflammation, goblet cell metaplasia and AHR (Bryce *et al* 2003). In humans however, although H_1 receptor antagonism offers symptomatic relief of several physiological events such as oedema and vasodilatation, the use of antihistamines has been limited due to their inability to diminish early and late phase bronchoconstriction responses, hyperreactivity and inflammation. For this reason, recent research has focused on the contribution of the additional histamine receptors in the symptoms of asthma.

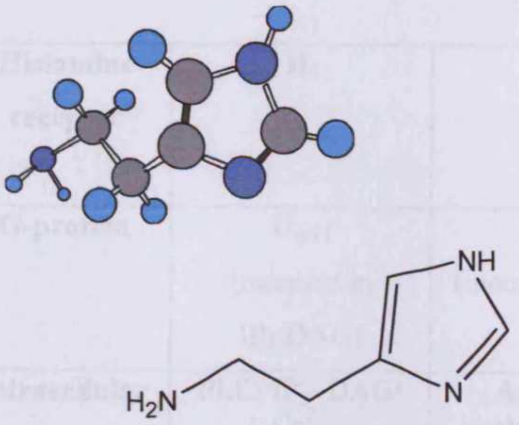
Although H_2 receptors mediate multiple effects throughout the body, they are not considered as important in the asthmatic response as the H_1 receptor. Arguably, the most important H_2-mediated biological action is its secretory effect in the airways and the gut. Histamine-induced goblet cell degranulation has been revealed in isolated human colonic crypts of Lieberkuhn (Halm and Halm 1999) and in the guinea pig trachea *in vivo* (Tamaoki *et al* 1997). H_2 receptor stimulation can increase cardiac output and heart rate (Tucker *et al* 1975) and mediate vasodilatation in some species but not humans (Wong *et al* 2004). H_2 receptors may also play a protective role in asthma, mediating airway smooth muscle relaxation in some species (Chen *et al* 1995).

The H_3 and H_4 receptors are the most recent of the histamine receptors to be identified and for this reason are not as well characterised as the H_1 and H_2 receptors. The H_3 receptor is located on presynaptic membranes and activation results in the release of histamine as well as additional neurotransmitters such as noradrenaline, dopamine, serotonin and acetylcholine. The H_3 receptor may also have a H_1 potentiating and H_2 antagonising role (Akdis and Blaser 2006). H_4 receptor mRNA is found in cells of the immune system including eosinophils, T cells and dendritic cells (Gantner *et al* 2002), while the H_4 receptor is expressed in the lung, on mast cells, fibroblasts, smooth muscle cells and epithelial cells, suggesting a potential role in airway allergic disease (Lippert *et al* 2004, Gantner *et al* 2002). It is important in mediator release and chemotaxis of inflammatory cells such as eosinophils, mast cells, dendritic cells and T cells. H_4 receptor deficient mice reveal reduced cytokine production following T cell activation, diminished eosinophilia and reduced inflammation following OA challenge (Dunford *et*

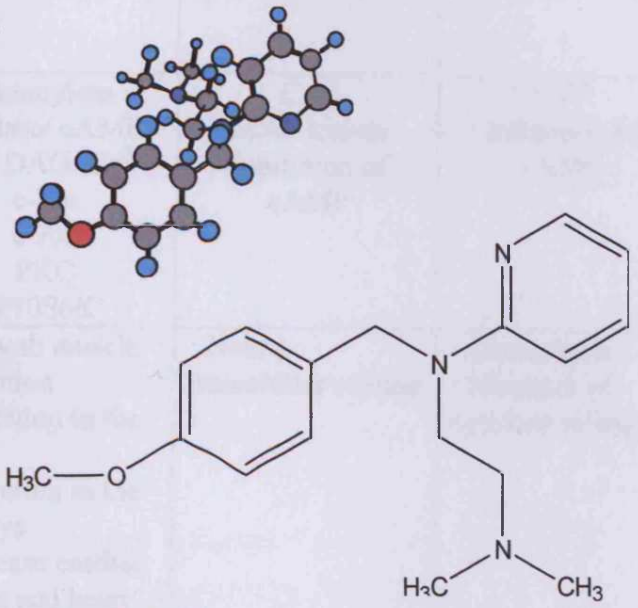
al 2006). These observations suggest the role of the H_4 receptor in autoimmune diseases such as asthma (Zhang *et al* 2006).

6.1.4 HISTAMINE RECEPTOR ANTAGONISTS

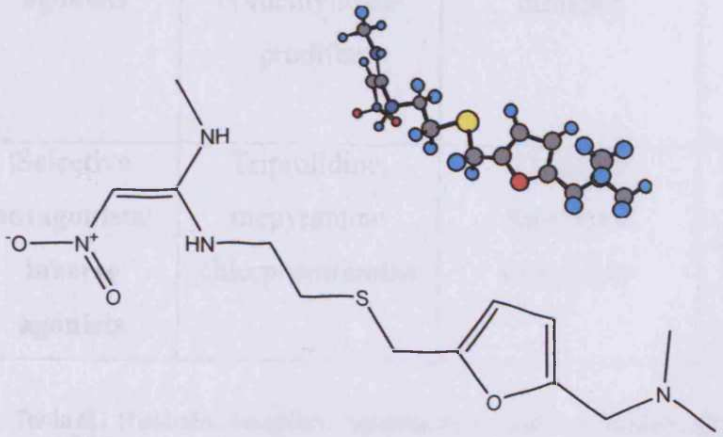
In the 1930s, Bovet described the first compounds that demonstrated antihistaminergic activity, which included piperoxan, aryl ethers and aniline ethylene diamine derivatives. One of the first antihistamines used in man was phenbenzamine. This was quickly replaced by mepyramine and followed by several other antihistamines including diphenhydramine, tripelennamine, chlorpheniramine and promethazine. All of these compounds were antagonists of the H_1 receptor and were found to alleviate some of the symptoms of allergy and inflammation. However, these first generation antihistamines are lipid soluble, allowing access across the blood brain barrier and side effects including sedation (promethazine), antimuscarinic effects and anti-emetic properties (cyclizine). Subsequent research resulted in the development of newer antihistamines such as loratidine and cetirizine, which are non-sedative (Parsens and Ganellin 2006).
The discovery that H_2 receptors had a significant effect on gastric acid secretion led to intense investigation into the development of an H_2-selective antagonist for the treatment of peptic ulcer disease. Cimetidine was the first H_2 receptor antagonist used clinically for peptic ulcer disease and this was followed by the further development of H_2 receptor antagonists including famotidine, roxatidine and ranitidine (for structure, see Fig.6.1). Antagonists for the H_3 and H_4 receptors have been developed mostly for research purposes, but there is considerable overlapping activity of these compounds at both receptors due to the similar homology of the H_3 and H_4 receptor (Parsons and Ganellin 2006). Some selective agonists and antagonists for all histamine receptors are listed in Table 6.1.



a. histamine



b. mepyramine



c ranitidine

Figure 6.1. Chemical structures of a) histamine, b) mepyramine and c) ranitidine.

Histamine receptor	H_1	H_2	H_3	H_4
G-protein	$G_{q/11}$ (increase in IP_3/DAG)	$G\alpha_s$ (increase cAMP)	$G_{i/o}$ (decrease cAMP)	$G_{i/o}$ (decrease cAMP)
Intracellular signalling	PLC/ IP_3/ DAG/ Ca^{2+} cGMP phospholipase D phospholipase A_2 PKC NF-κB	Adenylate cyclase/ cAMP IP_3/ DAG/ Ca^{2+} c-Fos c-Jun PKC P70S6K	Ca^{2+} MAP kinase Inhibition of cAMP	Ca^{2+} Inhibition of cAMP
Effects	· Vasodilation · Broncho-constriction · Activation of sensory nerves · Microvascular leakage · Ileum smooth muscle contraction	· Smooth muscle relaxation · Secretion in the gut · Secretion in the airways · Increase cardiac output and heart rate · Vasodilation	· Neuro-transmitter release	· Chemotaxis · Mediator of cytokine release
Selective agonists	Histaprodifen, N-methylhista-prodifen	Amthamine dimaprit	Immethridine N-Methyl-immepip	Clobenpropit 4-methyl-histamine Clozapine
Selective antagonists/ inverse agonists	Triprolidine, mepyramine chlorpheniramine	Tiotidine Ranitidine cimetidine	Clobenpropit, Thioperamide Ciproxifan	JNJ7777120 Thioperamide

Table 6.1 Histamine receptors. Adapted from Akdis and Blaser (2003) and SigmaAldrich 2007.

6.2 AIMS AND OBJECTIVES

HYPOTHESIS. *In a chronically OA challenged guinea pig, a nebulised exposure of the secretagogue, histamine, induces mucus secretion and reductions in lung function.*

6.2.1 AIM

The aim of this chapter was to utilise plethysmography and histological methods to analyse the effect of nebulised histamine exposures on lung function responses and goblet cell-associated mucin secretion.

6.2.2 OBJECTIVES

- To demonstrate possible changes in lung function measurements subsequent to increasing doses of a single nebulised histamine exposure, in the presence of the H_1 antagonist mepyramine, in chronically OA challenged guinea pigs.

- To identify the effect of the H_2 receptor antagonist ranitidine on possible changes in lung function measurements following histamine exposure.

- To demonstrate whether nebulised histamine exposures induces epithelial mucin release, as measured by the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs.

- To identify the effect of the H_2 receptor antagonist ranitidine on possible changes in the mean % of AB/PAS-positive bronchiolar epithelial area following nebulised histamine exposures.

- To compare lung function responses following nebulised UTP exposure, histamine exposure or combined UTP and histamine exposures.

- To compare the effects of a nebulised UTP exposure, histamine exposure or combined nebulised exposure of histamine and UTP on airway mucus secretion.

6.3 METHODS

Groups of 6 male Dunkin-Hartley guinea pigs (supplied by Harlan, UK) weighing between 200-250g were used for all protocols.

6.3.1 SENSITISATION

Animals were sensitised on days 1 and 5 with an i.p, bilateral injection of a suspension containing 100 μg of OA and 100 mg aluminium hydroxide.

6.3.2 CHRONIC OA CHALLENGE

14 days following sensitisation (day 15), animals were exposed to a single nebulised solution of low dose OA (0.01% for 1hr). Animals were subsequently exposed to a nebulised solution of high dose OA (0.1% for 1hr) on days 17, 19, 21, 23, 25, 27 and 29. Mepyramine (30mg/kg) was administered by bilateral, i.p. injection 30mins prior to UTP exposure on days 17, 19, 21, 23, 25 and 27. For all OA challenges, a Wright nebuliser was used to supply air at a pressure of 20p.s.i. and at a rate of 0.3ml/min into a sealed stainless steel exposure chamber (40cm diameter, 15cm height). If any animal appeared in distress, the animal was removed from the exposure chamber and challenge considered complete.

6.3.3 EXPOSURES TO INDUCE MUCUS SECRETION

Subsequent to chronic OA challenge, guinea pigs were exposed to varying concentrations and lengths of nebulised histamine, nebulised UTP or combined histamine and UTP exposure to potentially induce goblet cell associated mucin secretion.

6.3.3.1 Nose-only histamine exposures

23hr 45mins following the last chronic OA challenge, animals were exposed to a single nose-only nebulised solution of histamine (1mM for 20secs). Guinea pigs were placed in a small animal restrainer consisting of high sides and a neck restrainer. A Wright

nebuliser was used to supply nebulised histamine at air pressure of 20p.s.i. and at a rate of 0.3ml/min through a tunnel with mouthpiece attached (see diagram 2.1). Lung function measurements were taken immediately prior to histamine exposure and subsequent to exposure at 0, 5 and 10mins.

6.3.3.2 Box histamine and UTP exposures

Animals were exposed to a single histamine (10mM for 30mins or 100mM for 30mins) or vehicle exposure (30mins) 22hrs 30mins following chronic OA challenge, or a single UTP (1mM for 15mins) or vehicle exposure (15mins) 22hrs 45mins following the last chronic OA challenge. For combined histamine and UTP exposures, animals were exposed to a single nebulised solution of histamine (10mM for 15mins) and a consecutive exposure of combined UTP and histamine (1mM and 10mM respectively for 15mins) 22hrs 30mins following chronic OA challenge. For all exposures, a Wright nebuliser was used to supply air at a pressure of 20p.s.i. and at a rate of 0.3ml/min into a sealed perspex chamber (15x 15x 32cm). Lung function measurements were taken immediately prior to histamine exposure and every 15mins up to 1hr subsequent to histamine exposure.

6.3.3.3 Antagonists

Mepyramine maleate (30mg/kg) was dissolved in saline and administered by i.p. bilateral injection 30mins prior to all box histamine exposures, but not the nose-only histamine exposure. Ranitidine (10mg/kg) was dissolved in saline and administered by i.p. bilateral injection 30mins prior to histamine exposure.

6.3.4 LUNG FUNCTION MEASUREMENTS

Whole body plethysmography was used to measure specific airway conductance (sG_{aw}) as previously described (Chapter 2).

6.3.5 TOTAL AND DIFFERENTIAL CELL COUNTS

24hrs subsequent to chronic OA challenge animals were terminated by a lethal overdose of sodium pentobarbitone and lungs lavaged. Total cells and differential cell counts (per

ml of lavage fluid) were determined using a Neubauer haemocytometer and cytospin smears, as previously described (Chapter 2).

6.3.6 HISTOLOGICAL ANALYSIS OF GUINEA PIG LUNGS

Immediately following lavage, lungs were removed from the thoracic cavity and fixed with formaldehyde. 3-5mm tangentially sliced portions of lung were processed into wax blocks, sectioned (3μm) using a Leica microtome and fixed onto glass slides. Slides were stained with AB/PAS and Mayers haemolum and each bronchiole analysed to give the % of AB/PAS-positive area of the bronchiolar epithelium. This was calculated for each bronchiole and mean values calculated. Detailed methodology is described in Chapter 2.

6.4 RESULTS

6.4.1 THE EFFECT OF A NOSE-ONLY THRESHOLD DOSE OF NEBULISED HISTAMINE ON LUNG FUNCTION IN CHRONICALLY OA CHALLENGED GUINEA PIGS

In sensitised and acutely OA challenged guinea pigs, a threshold dose of inhaled histamine induces an immediate bronchoconstriction, which is quickly recovered (see Chapter 3). Fig. 6.2 represents the mean time course for changes in sG_{aw}, up to 10mins following a single nose-only threshold dose of histamine 24hrs prior to and 23hrs 45mins subsequent to chronic OA challenge in chronically OA challenged guinea pigs. In chronically OA challenged guinea pigs, a single threshold dose of inhaled histamine produced a significant, immediate bronchoconstriction (-15.62 ± 2.82 % reduction in sG_{aw} compared to baseline), which fully recovered within 10 minutes (Fig. 6.2).

6.4.2 EFFECT OF BOX EXPOSURE TO NEBULISED HISTAMINE ON LUNG FUNCTION RESPONSES IN CHRONICALLY OA CHALLENGED GUINEA PIGS.

In chronically OA challenged guinea pigs, a single nebulised histamine exposure (10mM for 30mins), 30mins subsequent to an i.p. injection of the H_2 antagonist mepyramine (30mg/kg), induced a prolonged reduction in sG_{aw} up to 1hr, significant at 45mins. However, attenuation of the histamine-induced reduction in lung function was revealed following pre-treatment with an i.p. injection of ranitidine (10mg/kg) 30mins prior to histamine exposure (Fig. 6.3).

To demonstrate whether the histamine-induced reduction in lung function was dose-dependent, chronically OA challenged guinea pigs were exposed to a 10fold higher concentration of nebulised histamine (100mM for 30mins) 30mins subsequent to an i.p. injection of mepyramine (30mg/kg). A single exposure of high dose histamine (100mM for 30mins) produced an immediate, significant reduction in lung function, which was recovered at 30mins followed by a later onset reduction in lung function, significant at 45 and 60mins (Fig. 6.4).

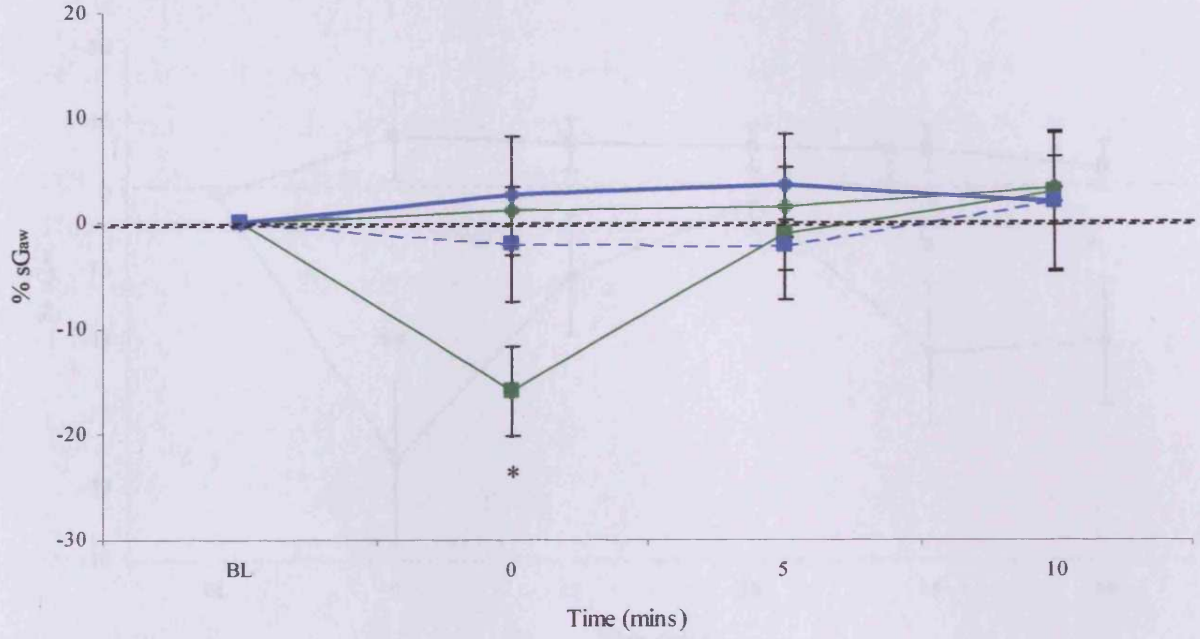


Figure 6.2. The effect of an inhaled nose-only nebulised exposure of histamine (1mM/20secs) 24hrs prior to (◆) and 23hrs 45mins subsequent to (■) chronic OA challenge on lung function measurements in sensitised guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. Lung function measurements after vehicle exposure (20secs) prior to (◆) and subsequent to (■) chronic OA challenge are also shown. n=4. * ($p<0.05$) significantly different from vehicle.

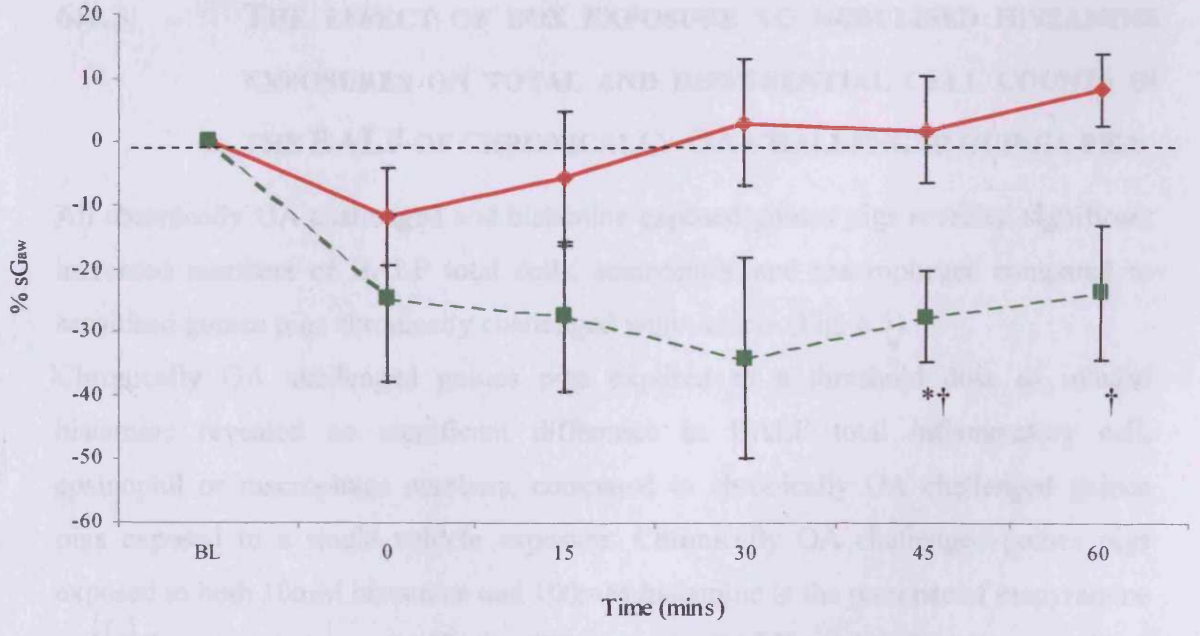


Figure 6.3. The effect of a single box histamine exposure (10mM/30mins), 1hr subsequent to an i.p. injection of either mepyramine (30mg/kg) and ranitidine (10mg/kg) (◆) or mepyramine (30mg/kg) and saline (1ml/kg) (■) on lung function in chronically OA challenged guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6. * ($p<0.05$) significantly different from baseline † ($p<0.05$) significantly different from ranitidine-treated guinea pigs.

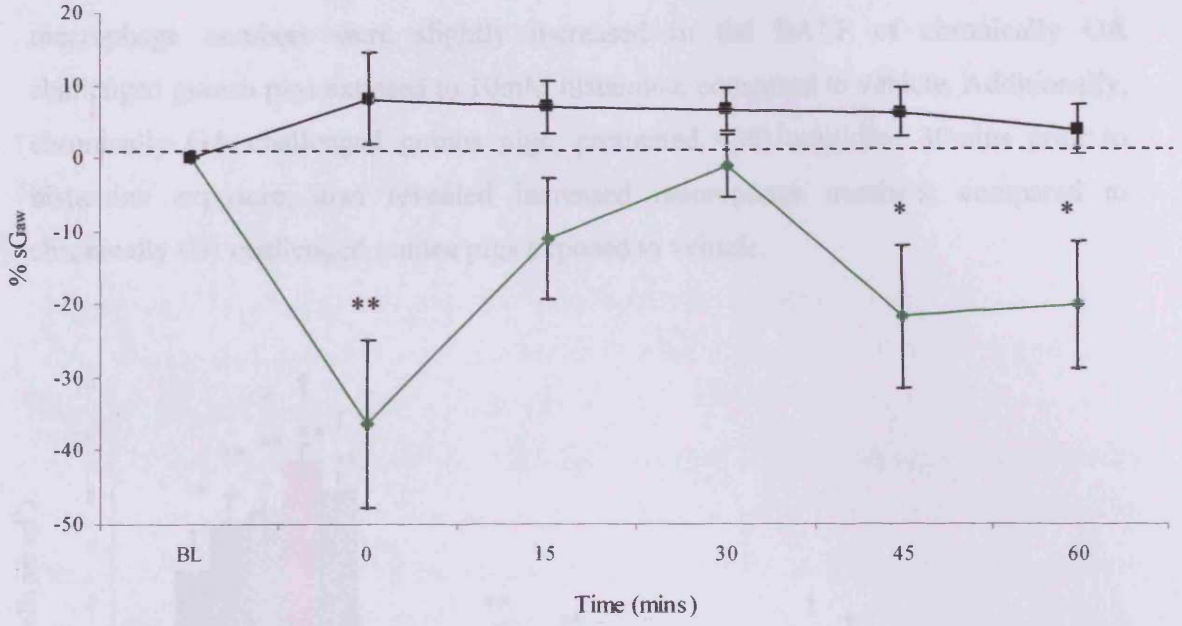


Figure 6.4. The effect of a single histamine (100mM/30mins) (◆) or vehicle exposure (30mins) (■), 30mins subsequent to an i.p. injection of mepyramine (30mg/kg) and saline, on lung function measurements in chronically OA challenged guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6. *($p<0.05$) ** ($p<0.01$) significantly different to vehicle

6.4.3 THE EFFECT OF BOX EXPOSURE TO NEBULISED HISTAMINE EXPOSURES ON TOTAL AND DIFFERENTIAL CELL COUNTS IN THE BALF OF CHRONICALLY OA CHALLENGED GUINEA PIGS

All chronically OA challenged and histamine exposed guinea pigs revealed significant increased numbers of BALF total cells, eosinophils and macrophages compared to sensitised guinea pigs chronically challenged with vehicle (Fig. 6.5).

Chronically OA challenged guinea pigs exposed to a threshold dose of inhaled histamine revealed no significant difference in BALF total inflammatory cell, eosinophil or macrophage numbers, compared to chronically OA challenged guinea pigs exposed to a single vehicle exposure. Chronically OA challenged guinea pigs exposed to both 10mM histamine and 100mM histamine in the presence of mepyramine and saline revealed no significant difference in BALF total inflammatory cell or eosinophil cell numbers compared to chronically OA challenged guinea pigs exposed to vehicle. However, although BALF macrophage numbers were not significantly different in chronically OA challenged guinea pigs exposed to 100mM histamine,

macrophage numbers were slightly increased in the BALF of chronically OA challenged guinea pigs exposed to 10mM histamine, compared to vehicle. Additionally, chronically OA challenged guinea pigs, pretreated with ranitidine 30mins prior to histamine exposure, also revealed increased macrophage numbers, compared to chronically OA challenged guinea pigs exposed to vehicle.

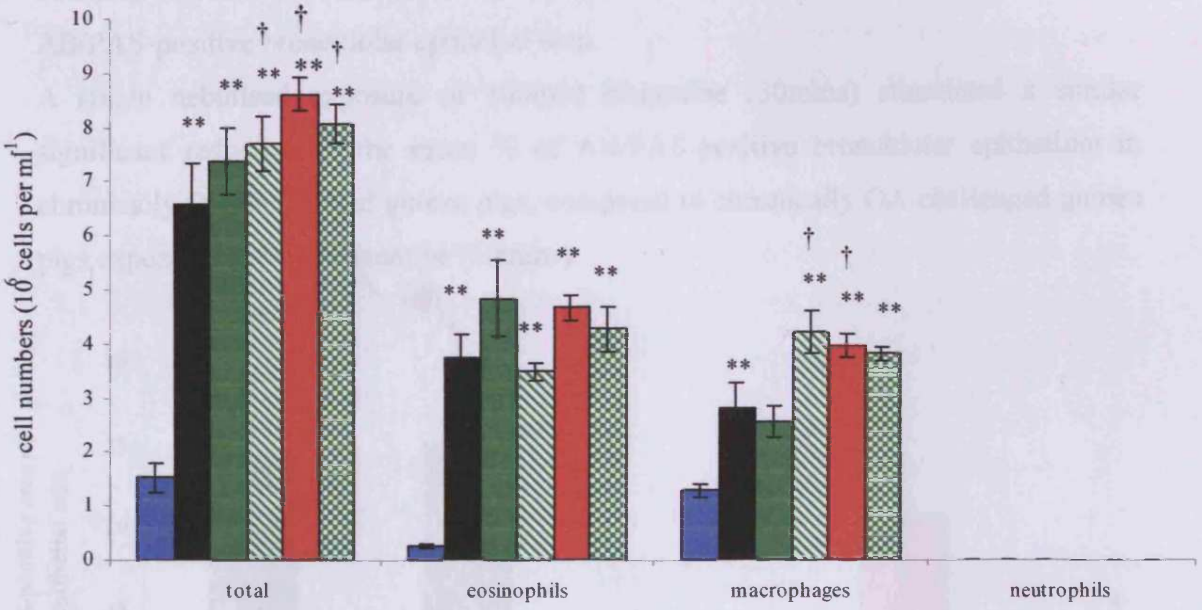


Figure 6.5. The effect of either chronic vehicle challenge (■) or one of the following exposure protocols on BALF total and differential cell numbers 24hrs subsequent to chronic OA challenge: 1) a single vehicle exposure (■). 2) a single nose-only histamine exposure (1mM/20secs) (■). 3) a single histamine exposure (10mM/30mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) and saline (▨). 5) a single histamine exposure (10mM/30mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) and ranitidine (10mg/kg) (■) or 6) a single histamine exposure (100mM for 30mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) and saline (▩). Results are expressed as mean ± s.e.m. cell numbers per ml of BALF.n=4,6. * ($p<0.05$) ** ($p<0.01$) significantly different to chronic vehicle (■). † ($p<0.05$) significantly different to vehicle (■)

6.4.4 EFFECT OF NEBULISED HISTAMINE EXPOSURES ON THE MEAN % OF AB/PAS-POSITIVE CELLS IN THE BRONCHIOLAR EPITHELIAL AREA OF CHRONICALLY OA CHALLENGED GUINEA PIGS

The mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs was not reduced following a single histamine exposure (1mM

for 20secs) compared to a single vehicle exposure, 23hrs 45mins subsequent to chronic OA challenge (Fig. 6.6). However, a single histamine exposure (10mM for 30mins), 30mins subsequent to an i.p. injection of mepyramine (30mg/kg) and saline induced a significant reduction in the mean % of AB/PAS-positive bronchiolar epithelial area in chronically OA challenged guinea pigs. An i.p. injection of ranitidine (10mg/kg) and mepyramine (30mg/kg), 30mins prior to a single histamine exposure (10mM for 30mins) revealed attenuation of the histamine-induced reduction in the mean % of AB/PAS-positive bronchiolar epithelial area.

A single nebulised exposure of 100mM histamine (30mins) stimulated a similar significant reduction in the mean % of AB/PAS-positive bronchiolar epithelium in chronically OA challenged guinea pigs, compared to chronically OA challenged guinea pigs exposed to 10mM histamine (30mins).

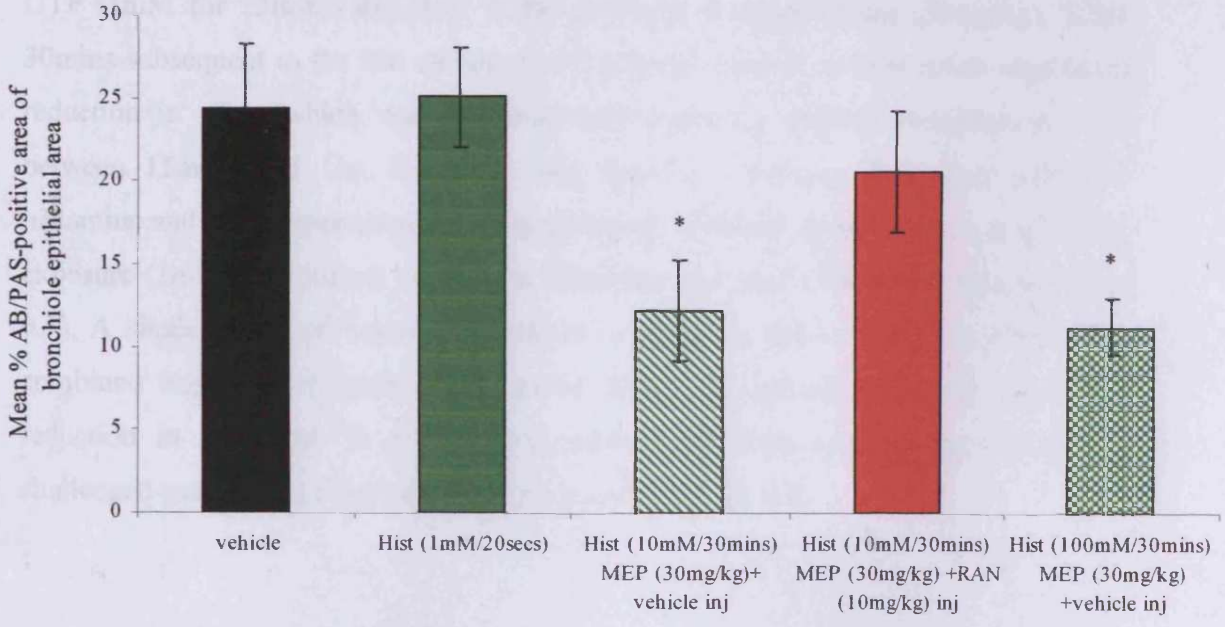


Figure 6.6. The mean % of AB/PAS-positive bronchiolar epithelial area, 24hrs subsequent to chronic OA challenge, in chronically OA challenged guinea pigs exposed with one of the following protocols: 1) a single vehicle exposure (30mins) (■). 2) a single nose-only histamine exposure (1mM/20secs) (■). 3) a single histamine exposure (10mM/30mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) and saline (▨). 5) a single histamine exposure (10mM/30mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) and ranitidine (10mg/kg) (■) or 6) a single histamine exposure (100mM for 30mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) and saline (▨). Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of tangentially sliced guinea pig left lung. n=6. * ($p<0.05$) significantly different to vehicle

6.4.5 THE EFFECT OF A COMBINED HISTAMINE AND UTP NEBULISED EXPOSURE ON LUNG FUNCTION AND THE MEAN % OF AB/PAS-POSITIVE BRONCHIOLAR EPITHELIAL AREA

It was previously proposed that nebulised exposure to UTP (1mM for 15mins) induced a gradual reduction in lung function, as a result of goblet cell-associated mucin secretion. Therefore, the effect of combined UTP and histamine exposure on lung function responses and % of AB/PAS-positive bronchiolar epithelial area was assessed to determine whether increased goblet cell mucin secretion and a further reduction in lung function could be induced. A 10mM (30mins) histamine exposure and a 1mM (15mins) UTP exposure, previously induced a maximum reduction in epithelial stored mucin (Fig. 6.6 and Fig. 4.9 respectively). These concentrations were therefore chosen for combined secretagogue exposure. A combined histamine (10mM for 30mins) and UTP (1mM for 15mins) exposure in the presence of mepyramine (30mg/kg), 22hrs 30mins subsequent to the last chronic OA challenge induced an immediate significant reduction in sG_{aw}, which was alleviated and reached a plateau reduction in sG_{aw} between 15mins and 1hr. However, lung function responses following combined histamine and UTP exposure were not significantly different compared to a single UTP exposure (1mM for 15mins) or a single histamine exposure (10mM for 30mins) (Fig. 6.7). A single nebulised histamine exposure, a single nebulised UTP exposure and a combined exposure of nebulised histamine and UTP induced a similar significant reduction in the mean % of AB/PAS-positive epithelial area in chronically OA challenged guinea pigs compared to vehicle exposure (Fig. 6.8).

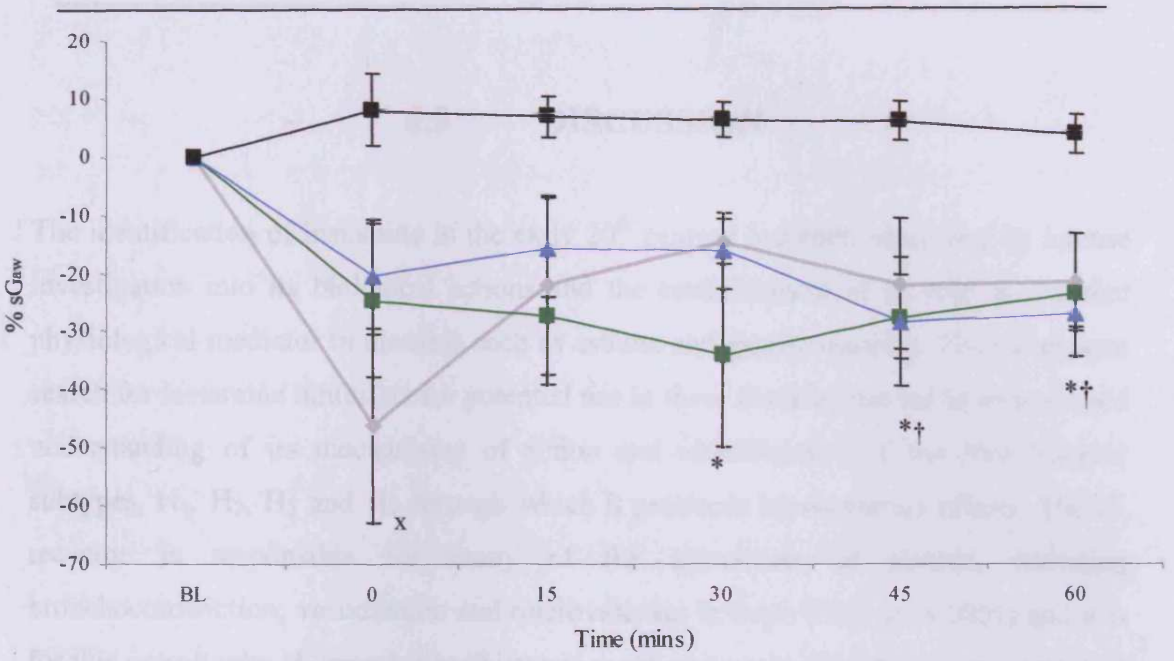


Figure 6.7. The effect of a single nebulised vehicle (30mins) exposure (■), a single nebulised UTP (1mM/15mins) exposure (◆), a single histamine (10mM/30mins) exposure (■) 30mins following an i.p. injection of mepyramine maleate (30mg/kg) and saline, or a combined UTP (1mM/15mins) and histamine (10mM/30mins) exposure (◇) 30mins following an i.p. injection of mepyramine maleate (30mg/kg) on lung function measurements in chronically OA challenged guinea pigs. Each point represents the mean ± s.e.m. change in sG_{aw} compared to baseline sG_{aw} values. n=6. * † x ($p<0.05$) significantly different (UTP, histamine and combined exposure respectively) to vehicle control.

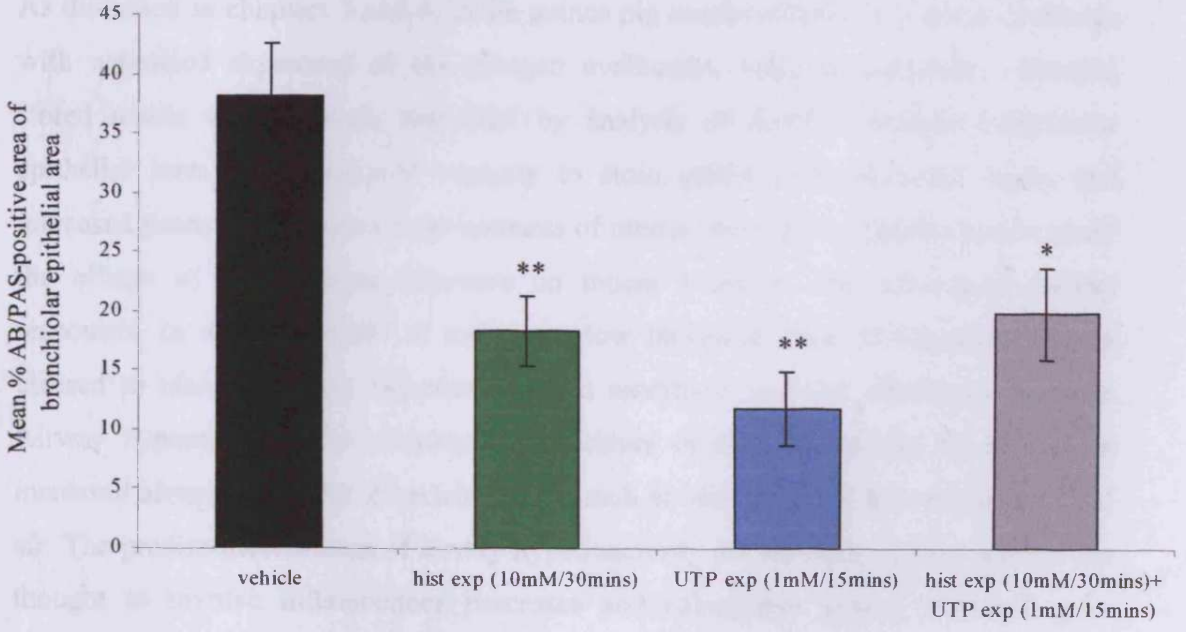


Figure 6.8. The mean % of AB/PAS-positive bronchiolar epithelial area, 24hrs subsequent to chronic OA challenge, in chronically OA challenged guinea pigs exposed with one of the following protocols: 1) a single box vehicle exposure (■). 2) a single histamine exposure (10mM/30mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) and saline (■). 3) a single UTP exp (1mM/15mins) (■) or 4) a combined histamine exposure (10mM /30mins) and UTP exp (1mM/15mins) 30mins subsequent to an i.p injection of mepyramine (30mg/kg) (■). Each point represents the mean % of AB/PAS-positive bronchiolar epithelial area in sections (3μm) of tangentially sliced guinea pig left lung. n=6. * ($p<0.05$) ** ($p<0.01$) significantly different to vehicle.

6.5 DISCUSSION

The identification of histamine in the early 20th century has been succeeded by intense investigation into its biological actions and the establishment of its role as a potent physiological mediator in diseases such as asthma and gastric ulceritis. The subsequent search for histamine inhibitors for potential use in these diseases has led to an increased understanding of its mechanisms of action and identification of the four receptor subtypes, H_1, H_2, H_3 and H_4, through which it produces its numerous effects. The H_1 receptor is responsible for many of the symptoms of asthma, including bronchoconstriction, vasodilation and microvascular leakage (Hart *et al* 2001) and it is for this reason why H_1 receptor antihistamines are an important target for the treatment of allergic disease. However in the airways, goblet cell and submucosal gland mucin secretion can also be mediated via H_2 receptors (Tamaoki *et al* 1997), which are located throughout the airway epithelium. It is this histaminergic property that has been exploited in this chapter.

As discussed in chapters 3 and 4, in the guinea pig model of asthma, chronic challenge with nebulised exposures of the allergen ovalbumin, induced significant epithelial stored mucin accumulation, measured by analysis of AB/PAS-stained bronchiolar epithelial area. This increased capacity to store goblet cell-associated mucin and increased potential to secrete large amounts of mucus provided a valuable tool to study the effects of secretagogue exposure on mucin secretion and subsequent airway responses. In animal models of asthma, a low threshold dose of histamine can be utilised to identify airway hyperreactivity in sensitised and OA challenged animals. Airway hyperreactivity is a characteristic feature of asthma and can be defined as increased airway reactivity to certain stimuli such as histamine, ACh, exercise and cold air. The precise mechanisms of airway hyperreactivity are not fully understood but are thought to involve inflammatory processes and subsequent airway remodelling in response to allergen challenge (O'Bryne and Inman 2003). A nebulised exposure of a threshold dose of histamine in acutely OA challenged guinea pigs and chronically OA challenged guinea pigs induced an immediate bronchoconstriction which was quickly recovered (Figs. 3.2 and 6.2). However, this dose of histamine was not associated with a

reduction in epithelial stored mucin in chronically OA challenged animals (Fig. 6.6) suggesting that an increased dose of histamine is required to stimulate mucus secretion.

In chronically OA challenged guinea pigs, a single box exposure to nebulised inhalation of histamine (10mM for 30mins), in the presence of mepyramine stimulated a gradual and prolonged reduction in lung function, significant at 45mins. Furthermore, histological analysis revealed a significant reduction in goblet cell-associated mucin in these animals, 1hr subsequent to histamine exposure, suggesting histamine-induced airway mucin secretion.

Activation of H_1 receptors can induce both bronchoconstriction and possible goblet cell mucus secretion via ACh release following activation of H_1 receptors on cholinergic nerve terminals (Takeyama *et al* 1996). An i.p. injection of mepyramine prior to inhaled histamine was essential in order to protect against fatal anaphylaxis. Mepyramine maleate is an H_1 receptor antagonist and it can therefore be concluded that lung function responses to inhaled histamine was not a result of direct H_1-mediated bronchoconstriction or indirect H_1-receptor mediated mucus secretion via muscarinic receptor stimulation and ACh release.

Both histamine-induced responses appeared to be mediated via the H_2 receptor, as pretreatment with the H_2 receptor antagonist ranitidine inhibited lung function responses and attenuated histamine-induced mucin secretion. Activation of the H_2 receptor can stimulate several effects in the airways. H_2 receptor-mediated bronchodilation has been observed in rats, cats, rabbits, sheep and horses (Akdis and Blaser 2003). However, H_2 receptor-mediated bronchodilation has not been revealed in humans and nebulised histamine did not induce bronchodilation in our guinea pig model. H_2-mediated goblet cell mucin secretion has previously been demonstrated in several experimental models, including the guinea pig. Tamaoki *et al* (1997) revealed H_2-mediated airway goblet cell secretion in guinea pig trachea. The present results suggest that in the chronically OA challenged guinea pig, a single exposure of inhaled histamine (10mM for 30mins) stimulates goblet cell mucin secretion via H_2 receptors, resulting in airway mucus accumulation and a subsequent reduction in lung function.

Although histamine-induced mucus secretion is attenuated with ranitidine pretreatment, it is not completely abolished. This may be due to incomplete inhibition of the H_2 receptor following a 10mg/kg dose of ranitidine. Additionally, activation of the H_3 or H_4

receptor may partially contribute to the goblet cell mucus secretion. H_4 receptor mRNA is found in cells of the immune system including eosinophils, T cells and dendritic cells (Gantner *et al* 2002), while the H_4 receptor is expressed in the lung, on mast cells, fibroblasts, smooth muscle cells and epithelial cells, suggesting a potential role in airway allergic disease (Lippert *et al* 2004, Gantner *et al* 2002). Further investigation using specific H_3 or H_4 receptor antagonists could deduce whether either the H_3 or H_4 receptor are involved in histamine-induced goblet cell mucus secretion in the airways.

Two phases of lung function responses were observed following a nebulised exposure of histamine at a higher concentration (100mM): an immediate and quickly recovered reduction in sG_{aw}, followed by a gradual and prolonged reduction in sG_{aw} significant at 45mins and 60mins. This was observed in association with a similar reduction in the mean % of AB/PAS-positive bronchiolar epithelial area, compared to that revealed following a 10mM histamine exposure. Thus, the immediate reduction in sG_{aw} appears to be a result of H_1-mediated bronchoconstriction, following inadequate mepyramine protection and that the later reduction in sG_{aw} is due to H_2 receptor mediated mucus secretion. Furthermore, 10mM and 100mM nebulised histamine induced similar lung function responses at 45 and 60mins and comparable reductions in the % of AB/PAS-positive bronchiolar epithelial area, suggesting equivalent mucin secretion and a maximum effect at both doses.

Nebulised UTP and histamine exposures induce goblet cell degranulation and subsequent mucin secretion via two different receptors, the $P2Y_2$ receptor and H_2 receptor respectively. A single exposure of inhaled UTP (1mM for 15mins) or inhaled histamine (10mM for 30mins) induced comparable lung function responses and goblet cell mucin secretion in chronically OA challenged guinea pigs (Fig. 6.7). However, although a cumulative increase in goblet cell mucin secretion may be expected following a combined exposure of nebulised UTP and histamine, compared to a single secretagogue exposure, this was not observed in these studies. Additionally, a combined exposure of nebulised UTP and histamine induced similar reductions in the mean % of AB/PAS-positive bronchiolar epithelial area compared to a single UTP or histamine nebulised exposure. This suggests that although goblet cell-associated mucin secretion may be stimulated via 2 separate mechanisms, a maximum volume of goblet cell-associated mucin secretion may be stimulated via either mechanism.

Sensitised guinea pigs reveal increased total and differential cell infiltration into the airways in response to chronic OA challenge (Chapter 3). However, total and differential inflammatory cell numbers in guinea pig BALF was not affected by a single threshold dose of inhaled histamine in chronically OA challenged guinea pigs. Inhaled histamine at higher doses (10mM and 100mM) however, revealed increased BALF macrophage numbers at 1hr subsequent to histamine exposure, compared to vehicle exposed animals, suggesting a potential role for histamine in macrophage chemotaxis via the H_3 or H_4 receptor. However, although histamine may play a role in eosinophil chemotaxis (Ling *et al* 2004), to date, and to my knowledge, there is no evidence for a role of histamine in macrophage recruitment to the airways. Alternatively, stimulation of excessive mucus production and increased bronchoconstriction during the chronic OA challenge and histamine exposure may result in inadequate cell retrieval during the BAL procedure and therefore inaccurate cell counts.

In conclusion, high doses of nebulised histamine induce similar airway responses as nebulised UTP. A high dose of histamine stimulated a H_2-mediated prolonged reduction in lung function associated with a significant reduction in epithelial stored mucin. It is therefore proposed that the histamine exposure can induce goblet cell mucin secretion, resulting in mucus accumulation in the airways and a subsequent reduction in lung function.

CHAPTER 7

Validation of a lectin-based assay for the quantification of guinea pig airway mucin

7.1 INTRODUCTION

Mucus hypersecretion is ubiquitously associated with respiratory inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). It can result in airway mucus accumulation and contribute to airway obstruction. Although there has been substantial research investigating the mechanisms behind the development of a mucus hypersecretory phenotype, there has been little research into the effect of mucus hypersecretion on lung function. In previous chapters, the effect of chronic allergen exposure on lung function and epithelial mucin content in sensitised and challenged guinea pigs was investigated. Both reduced daily baseline lung function measurements and increased goblet cell-associated stored mucin was demonstrated following chronic OA challenge, compared to a single OA challenge (Figs. 3.1.1, 3.2.1). Goblet cell-associated mucin content can be assessed by histological and morphometric analysis of stained guinea pig paraffin lung sections (Section 2.3). Various cellular components and tissue elements within the airways are identified using specific stains. For example, alcian blue/periodic acid Schiff (AB/PAS) is widely used for the positive identification of mucin in the airways of several laboratory animals (Sueyashi *et al* 2004, Komori *et al* 2001). The different binding profiles of AB and PAS permits differentiation between acidic mucins that stain blue (from AB) and neutral mucins that stain magenta (from PAS). However, due to the lack of specificity, general proteins found in many cell types and tissue components such as connective tissues and basal laminae are also identified using AB/PAS. Despite this, the distinct morphology (goblet-shaped) and specific location of goblet cells within the epithelial layer of the airways allow the identification of goblet cell-associated mucin following staining with non-specific stains such as AB/PAS. The development of methods allowing detection and quantification of mucins present in the airway lumen is critical for future understanding of mucus hypersecretion in respiratory disease. However, for efficient fixation of tissue and effective identification of goblet cell structures in the airway epithelium, inflation of lungs is essential. A consequence of this process is opening of the airway lumen and subsequent difficultly in identifying mucus plugs or bronchiolar mucus accumulation.

Bronchoalveolar lavage (BAL) is a diagnostic procedure involving instillation of saline into the airways and its subsequent removal (See Section 2.2.5 for detailed methodology). Analysis of the resulting sample allows measurement of various parameters of airway inflammation, including inflammatory cell populations, antibody levels or inflammatory mediator concentrations. Bronchoalveolar lavage fluid (BALF) may also provide a means to quantify mucus in the airway lumen. However, the measurement of mucus in BALF poses two major experimental hurdles. Firstly, AB/PAS is the principle stain for the measurement of mucin in tissue sections. However, due to its non-specificity, AB/PAS may react with other proteins present in BALF, such as plasma proteins, proteoglycans or inflammatory cells. Therefore AB/PAS cannot be utilised as an effective tool for the quantification of BALF mucin. Secondly, few scientific papers detail adequate protocols for the measurement of mucin in biological samples. The few exceptions include Jefcoat *et al* (2001) who describe the use of an ELISA utilising monoclonal antibody 4E4 for the measurement of mucin in horse BALF and Phillips *et al* (2006) who incorporated AI0G5 and 45MI into an ELISA for the quantification of mucin concentrations in rat BALF. The utilisation of an ELISA for the measurement of guinea pig BALF mucin requires guinea pig specific anti-mucin antibody. However, commercially available mucin antibodies have not been well characterised and specificity towards guinea pig mucin is questionable.

An enzyme-linked lectin assay (ELLA) has previously been used to measure airway mucin in rat biological samples (Jackson *et al* 2002). The term lectin is used to describe a group of proteins found in plants, viruses, micro-organisms or animals that have a specific carbohydrate binding property. Individual lectins selectively bind specific oligosaccharides, depending on the presence and position of sugar residues, which can enable the use of lectins to identify particular biological structures or components (Vector Laboratories 2007). Mucins are high Mw glycoproteins consisting of a peptide backbone and o-glycosidic linked oligosaccharides (Rose *et al* 1992). Oligosaccharide sugar types, including N-acetylgalactosamine, N-acetylglucosamine, galactose, sialic acid and fucose, provide potential targets for specific lectin binding (Jefcoat *et al* 2001) (Fig. 7.1). Jackson *et al* (2002) exploited the lectin UEA-I,

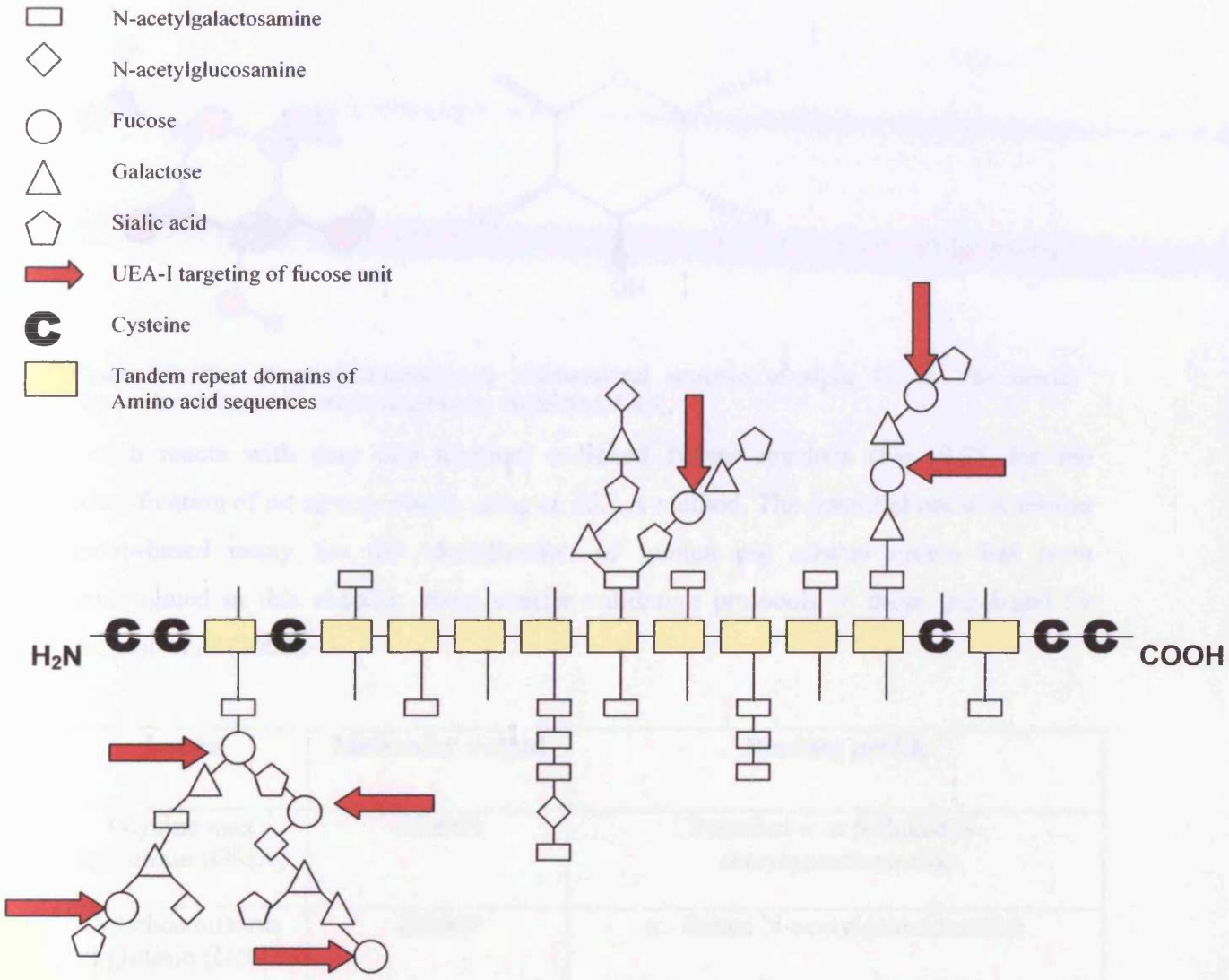


Figure 7.1. Theoretical structure of a secreted mucin unit and demonstration of fucose targeting by the lectin, UEA-I.. The peptide backbone of mucin is composed of tandem repeat domains of amino acid sequences and cysteine rich domains at the amino and carboxyl termini, which provide sites for o-linked glycosylation and disulphide bonds for oligomerisation respectively. Oligosaccharide units consist of different combinations of the sugars, N-acetyl galactosamine, N-acetylglucosamine, sialic acid, fucose and galactose. Oligosaccharides can be linear, branched and can vary in size. Oligosaccharides are linked to the peptide backbone by o-glycosidic linkage between N-acetylgalactosamine and serine or threonine of the peptide repeat domains. The lectin UEA-I targets α-linked fucose units of the oligosaccharides (Voynow *et al* 2002, Rose *et al* 1992)

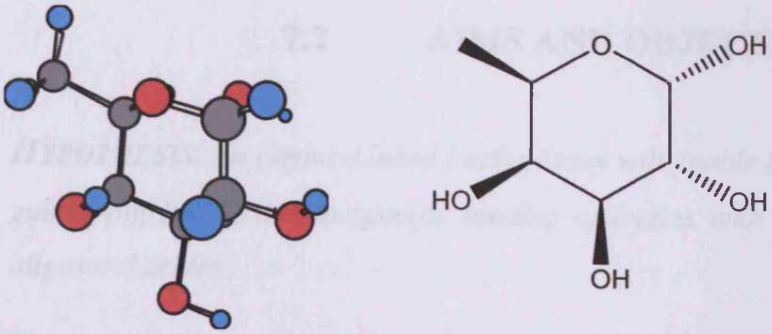


Figure 7.2. The chemical structure and 3-dimensional structure of alpha fucose. The mucin oigosaccharide sugar, fucose, is targetted by the lectin UEA-I

which reacts with core and terminal α–linked fucose residues (Fig. 7.2), for the identification of rat airway mucin using an ELLA method. The potential use of a similar lectin-based assay for the identification of guinea pig airway mucin has been investigated in this chapter, using similar validation protocols to those published by Jackson *et al* (2002).

Lectin	Molecular weight	Binding profile
Glycine max agglutinin (GMA)	120000	Terminal α or β-linked N-acetylgalactosamine
Dolichos biflorus agglutinin (DBA)	120000	α –linked N-acetylgalactosamine
Triticum vulgaris agglutinin (TVA)	36000	N-acetylglucosamine
Ulex europaeus agglutinin I (UEA-I)	63000	α-linked fucose residues
Vicia Villosa lectin (VVL)	139000	Terminal α or β-linked N-acetylgalactosamine
Helix pomatia agglutinin(HPA)	79000	α –linked N-acetylgalactosamine

Table 7.1. The binding profiles and Mws of individual lectins (Vector Laboratories 2007, SigmaAldrich 2007).

7.2 AIMS AND OBJECTIVES

HYPOTHESIS. *An Enyme-Linked Lectin Assay will enable the measurement of mucin in guinea pig BALF, due to specific binding of lectins with individual sugars of mucin oligosaccharides.*

7.2.1 Aim

The aim of the chapter was to validate a Sandwich Enzyme Linked Lectin Assay (Sandwich ELLA) for the measurement of mucin in guinea pig BALF.

7.2.2 Objectives

- Adoption of histological methods to evaluate the staining profile of a panel of lectins in comparison to staining with AB/PAS, in order to evaluate a goblet cell staining lectin (lectin candidate).

- To identify a lectin candidate with specific reactivity to high Mw proteins in BALF, using SDS-PAGE and Western blot analysis.

- To demonstrate insensitivity of the lectin candidate to the high Mw material proteoglycans hyaluronic acid and chondroitin sulphate, which may also be present in BALF.

- To demonstrate insensitivity of the lectin candidate to blood components, such as plasma proteins and blood cells.

- To optimise an ELLA for the quantification of mucin in guinea pig BALF

7.3 MATERIALS AND EQUIPMENT

7.3.1 Materials

Human mucus standard was provided by Novartis (Horsham, UK). Purified human mucin was supplied diluted at 1:2000 in 50% glycerol (Jackson *et al* 2002)

All other materials and reagents were supplied by Sigma unless otherwise stated.

7.3.2 Equipment

DeVilbiss Pulmostar Nebuliser (Sunrise Medical Ltd, Wollaston, UK)

Bio-dot apparatus (Bio-Rad Laboratories Ltd, Hercules, USA)

Denley BR401 Centrifuge (Denley, UK)

Hoefer TE Series Transphor EPS (38B12) SDS-PAGE Tank (Hoefer, San Francisco, USA)

Hoefer TE Series Transphor EPS Blotting Unit (Hoefer, San Francisco, USA)

Embla automated cell washer (Molecular Devices, Sunnyvale, USA)

SpectraMax 340PC Microplate Spectrophotometer (Molecular Devices Ltd, Sunnyvale, USA)

7.4 METHODS

7.4.1 Guinea pig treatments

Guinea pigs were sensitised to OA and treated with one of the following exposure protocols: a single exposure of low-dose OA (acutely challenged); a chronic exposure of high-dose OA (chronically challenged) or a chronic exposure of high-dose OA followed by nebulised UTP exposure (secretagogue challenged).

7.4.1.1 Sensitisation

All animals were sensitised on days 1 and 5 with an intra-peritoneal (i.p.), bilateral injection of a suspension containing 100 μg of OA and 100 mg aluminium hydroxide.

7.4.1.2 Acutely-challenged guinea pigs

14 days following sensitisation (day15), animals were exposed to a nebulised solution of OA (0.01% for 1hr) in a stainless steel exposure chamber (40cm diameter, 15cm height). A Wright nebuliser was used to supply air at a pressure of 20p.s.i and at a rate of 0.3 ml/min. If any of the animals looked in distress, they were withdrawn from the chamber and exposure considered complete. 24hrs following OA exposure guinea pigs were overdosed with an i.p. injection of sodium pentobarbitone (Euthatal 400mg/kg).

7.4.1.3 Chronically-challenged guinea pigs

14 days following sensitisation (day 15), animals were exposed to a single nebulised solution of low dose OA (0.01% for 1hr), using a Wright nebuliser, in a stainless steel exposure chamber (40cm diameter, 15cm height). Animals were subsequently exposed to a nebulised solution of high dose OA (0.1% for 1hr) on days 17, 19, 21, 23, 25, 27, and 29. Administration of the antagonist mepyramine (30mg/kg) by bilateral intra-peritoneal (i.p.) injection 30mins prior to high dose OA exposure on days 17-27 protected against fatal anaphylaxis. 24hrs following the chronic OA exposure period (day 30) guinea pigs were overdosed with an i.p. injection of sodium pentobarbitone (Euthatal 400mg/kg).

7.4.1.4 Secretagogue challenged guinea pigs

Guinea pigs were chronically OA challenged, as described in section 7.4.1.3. 22hrs 45mins following the chronic OA exposure period (day 30) guinea pigs received a nebulised exposure of UTP (1mM for 15mins). 1hr subsequent to UTP exposure, guinea pigs were overdosed with an i.p. injection of sodium pentobarbitone (Euthatal 400mg/kg).

7.4.2 Identification of a mucin staining lectin using histological analysis

Paraffin sections of guinea pig lung were prepared. Paired sections of lung were subsequently stained with either AB/PAS or lectin. Comparison of goblet cell-associated mucin staining by biotinylated lectin compared to AB/PAS allowed identification of a mucin staining lectin.

7.4.2.1 Preparation of guinea pig lung paraffin sections

Slices of guinea pig left lung were processed, set in wax and sectioned using a Leica microtome, as described in Sections 2.3.1, 2.3.2 and 2.3.3.

7.4.2.2 Histological staining of paraffin sections of guinea pig left lung: AB/PAS and lectin staining

Twelve sequential sections (3μm) of paraffin–embedded guinea pig left lung were divided into six adjacent pairs, one of which was stained with AB/PAS and the other with biotinylated lectin. This permits comparison of goblet cell-associated mucin staining by both stains.

7.4.2.2.1 AB/PAS staining

Slides were held in plastic slide racks and stained with AB/PAS and Mayer's haemalum using the following protocol:

1. Sections were dewaxed in xylene for 5mins and taken through graded IMS solutions (100%, 90% and 70%), each for 5mins.

2. Sections were washed in distilled water for 5mins.
3. Sections were immersed in 1% alcian blue in 3% aqueous acetic acid (pH 2.5) for 5mins.
4. Sections were rinsed in running tap water for 5mins.
5. Sections were immersed in periodic acid (0.5%) for 5mins.
6. Sections were rinsed in running tap water for 5mins.
7. Sections were washed in distilled water for 5mins.
8. Sections were immersed in Schiffs reagent (Surgipath) for 10mins.
9. Sections were rinsed in running tap water for 10mins.
10. Sections were dipped in Mayers haemalum for 20secs.
11. Sections were rinsed in running tap water for 5mins.
12. Sections were taken through graded IMS (70%, 90% and 100%) and cleared in xylene.
13. Sections were coverslipped.

7.4.2.2.2 Peroxidase method for staining with biotinylated lectins

1. Sections were dewaxed in xylene for 5mins and taken through graded IMS solutions (100%, 90% and 70%), each for 5mins.
2. Sections were placed in hydrogen peroxide (0.5%) in methanol for 10mins.
3. Sections were washed in running tap water for 5mins.
4. Sections were placed in distilled water at 37°C.
5. Sections were digested in a preparation of trypsin (0.1%) (ICN Pharmaceuticals Inc., USA) in calcium chloride (0.1%), pH7.8, 37°C for 15mins.
6. Digestion was stopped by rinsing the sections in running tap water for 5mins.
7. Sections were washed in Tris-buffered saline (TBS) (0.005M), pH7.6 for 5mins (x2).
8. Sections were incubated in bovine serum albumin (BSA) (0.1%) for 30mins.

9. Sections were drained.
10. Lectin solutions were diluted to required concentrations with DakoCytomation Antibody Diluent (DakoCytomation, Denmark), added to each section using a Pasteur pipette and left overnight at 4°C.
11. Sections were removed from the fridge and allowed to reach room temperature for 30mins.
12. Streptavidin-biotin-peroxidase (SABCpx) complex (DakoCytomation, Denmark) was prepared and allowed to stand for 30mins to complex.
13. Sections were washed with PBS for 5mins (x3).
14. SABCpx was applied to slides for 30mins.
15. Sections were washed with PBS for 5mins (x3).
16. Sections were incubated 3,3'-Diaminobenzidine (DAB) (0.5mg/ml) in TrisHCl buffer, pH7.6 and H_2O_2 (0.01%).
17. Sections were rinsed in running tap water for 5mins.
18. Sections were rinsed in distilled water and dipped in Cole's haemotoxylin (Surgipath Ltd, Europe) for 1min.
19. Sections were rinsed in running tap water for 5mins.
20. Haemotoxylin was differentiated by dipping sections in HCl (0.5%) in IMS (70%), and sections blued in running tap water for 5mins.
21. Sections were taken through various graded IMS solutions (70%, 90% and 100%) for 5mins and cleared in xylene for 5mins.
22. Sections were coverslipped.

7.4.2.3 Histological analysis and comparison of lectin-staining and AB/PAS staining of paraffin sections of guinea pig left lung.

Correlation of goblet cell-associated mucin staining by biotinylated lectin compared to AB/PAS staining was determined in paired sections by blind comparison, using both observational examination and analysis using a SigmaScan Image Analysis software program.

Acidic and neutral mucins were stained blue and magenta by AB and PAS respectively. Subsequent to AB/PAS staining, each bronchiole of guinea pig left lung sections were

photographed using a digital camera and microscope. The images were then analysed using SigmaScan Image Analysis program. A draw tool was used to trace the outline of the bronchiolar epithelium. The resulting area was used to determine the area of epithelium, expressed as numbers of pixels. A magenta/purple colour threshold measurement was then used to determine the area of epithelium that was positively stained with AB/PAS (expressed as number of pixels). The area of AB/PAS-stain positive cells was expressed as % of the total epithelial area.

Lectin-positive cells in the bronchiolar epithelium were analysed using the SigmaScan Image Analysis program, as described earlier for AB/PAS analysis. A black-brown colour threshold measurement was used to determine the area of lectin-stained bronchiolar epithelium.

7.4.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting of guinea pig BALF and subsequent staining with HRP-conjugated lectins

SDS-PAGE and Western blot are techniques used to separate and identify proteins in a given sample as a function of their Mw. Once separated, the proteins can be transblotted out of the gel onto a membrane. Subsequent staining of the membrane allows detection of stain-specific proteins of known Mws. SDS-PAGE and Western blot was used to identify the Mw of AB, PAS and lectin-stained proteins in samples of guinea pig BALF. The aim of this experiment was to identify a lectin, which specifically stained high Mw protein, possibly mucin, in BALF.

7.4.3.1 Preparation of samples for SDS-PAGE

1) Between 2 and 15ml of guinea pig BALF supernatant, previously removed of cells by centrifugation (6mins at 2000rpm, Mistral Centrifuge 3000, UK), was inserted into a centrifuge filter unit Ultrafree-15 (Millipore) and placed inside a standard centrifuge tube (25ml). To concentrate the BALF solution, the sample was centrifuged at 4000rpm until BALF sample was reduced 15fold.

2) 100µl of concentrated sample or human mucin standard was added to 80µl of Novex® Tris-Glycine SDS Sample buffer (Invitrogen Ltd, UK) and 20µl of NuPAGE® Sample Reducing Agent (Invitrogen Ltd, UK).

3) All samples were heated for 10mins at 100°C and immediately placed on ice, for at least 1min prior to loading of the gel.

4) SeeBlue® Plus 2 (Invitrogen Ltd, UK) was directly applied to the gel.

7.4.3.2 SDS-PAGE (gel loading and protein separation)

5) 4-20% Tris-Glycine Precast (1.0mm x 10-well) gels (Invitrogen Ltd, UK) were washed in distilled water and assembled in an XCell Sure Lock Mini Cell gel system (Novex®).

6) Both chambers of the system were filled with Tris-Glycine SDS Running Buffer (Novex®)(Invitrogen Ltd, UK), diluted from concentrated stock.

7) The well combs were removed from the gel and 0.5ml of antioxidant was added to the central chamber.

8) 10µl of SeeBlue® Plus 2 standard (Invitrogen Ltd, UK) and 15µl of all test samples were applied to designated wells using a 100µl Finn pipette and fine long tips.

9) The lid was placed in position and leads connected to corresponding electrodes.

10) The gels were run at constant amperage of 35mA with a maximum voltage of 125V for 2hrs or until the dye front advanced to the bottom slit of the gel cassette.

7.4.3.3 Western Blotting (Transblotting of protein to nitrocellulose membrane)

11) The polyvinylidene fluoride (PVDF) membrane was soaked in methanol for 5mins at room temperature prior to rehydration in protein transfer buffer (diluted with distilled water and methanol (20%)).

12) Protein Tris-Glycine Transfer Buffer (Novex®) (Invitrogen Ltd, UK), was added to the blotting electrophoresis tank to minimum fill line.

13) The gel was removed from its casing and rinsed in distilled water and placed in Tris-Glycine Transfer Buffer (Novex®) (Invitrogen Ltd, UK).

14) A sandwich struture consisting of a layer of sponge, filter paper (Invitrogen Ltd, UK), PVDF membrane (Invitrogen Ltd, UK), gel, a second filter paper and a second sponge (all pre-soaked in Tris-Glycine Transfer Buffer (Novex®)) was placed inside the sandwich cassette (Fig. 7.3)

15) Air bubbles were removed from between each layer by a gentle rolling motion with a 25ml pipette tip.

16) The cassette was clipped together and placed in the blotting tank, ensuring correct orientation.

17) The transfer unit was run at constant amperage of 125mA with a maximum voltage of 25V for 17hrs or overnight.

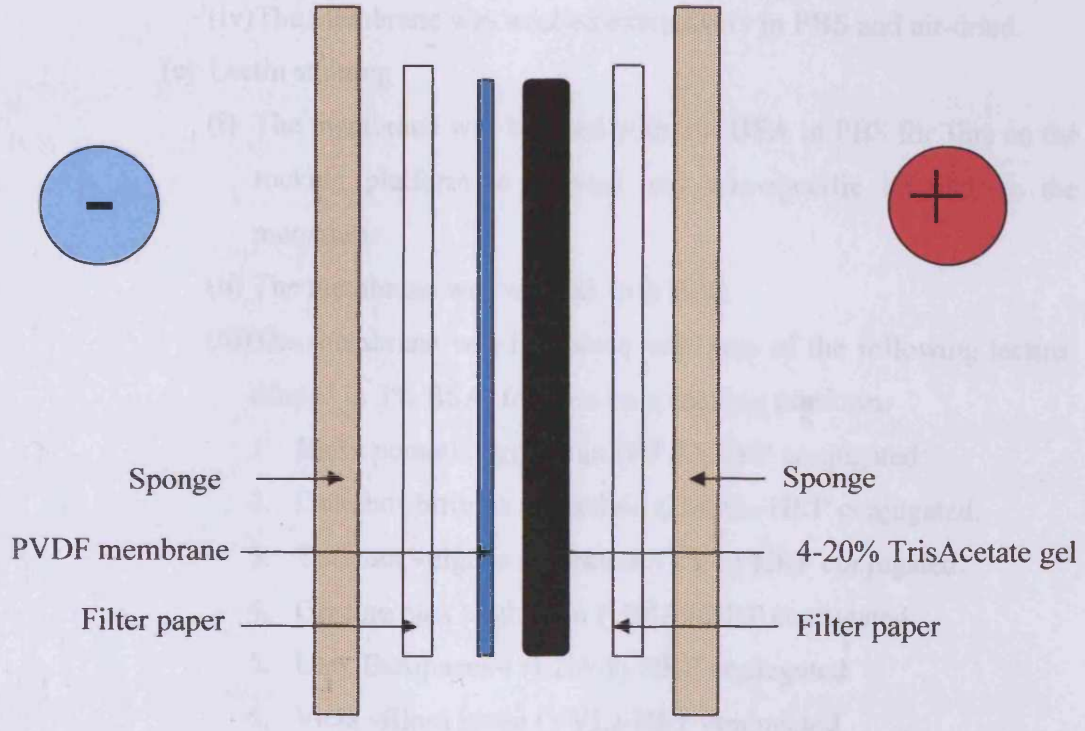


Figure 7.3. Positioning of equipment within the blot apparatus for Western blot protocol.

7.4.3.4 Staining of membrane with AB, PAS or lectin.

18) The membrane was removed from the sandwich and washed in PBS extensively.

19) The membrane was subsequently cut into designated sections and each section stained using one of the following staining protocols.

(a) Alcian blue (AB) staining

(i) The membrane was placed in AB (1%) (Surgipath Ltd, Europe) in aqueous acetic acid (3%) for 10mins.

(ii) The membrane was washed extensively in PBS and air-dried.

(b) Periodic acid /Schiff staining

(i) The membrane was placed in periodic acid (1%) for 10mins.

(ii) The membrane was washed with PBS for 10mins.

(iii)The membrane was placed in Schiff reagent (Surgipath Ltd, UK) for 10mins.

(iv)The membrane was washed extensively in PBS and air-dried.

(c) Lectin staining

(i) The membrane was blocked with 3% BSA in PBS for 3hrs on the rocking platform to prevent any non-specific binding on the membrane.

(ii) The membrane was washed with PBS.

(iii)The membrane was incubated with one of the following lectins, diluted in 3% BSA, for 2hrs on a rocking platform.

1. Helix pomatia agglutinin (HPA)-HRP conjugated.
2. Dolichos bifluros agglutinin (DBA) –HRP conjugated.
3. Triticum vulgaris agglutinin (TVA)-HRP conjugated.
4. Glycine max agglutinin (GMA)-HRP conjugated
5. Ulex Europaeus-I (UEA-I)-HRP conjugated
6. Vicia villosa lectin (VVL)-HRP conjugated

(iv)The membranes were washed in PBS.

(v) The membrane was added to 3,3'-diaminobenzidine (DAB) solution (0.2mg/ml) in PBS and H_2O_2 (0.01%) for 5mins or until staining was complete.

(vi) The membranes were washed extensively in PBS and air-dried.

7.4.4 SDS PAGE and Western blotting of digested guinea pig BALF and blood components: subsequent staining with the HRP-conjugated lectin, Ulex Europaeus (UEA-I)

The high Mw proteoglycans, hyaluronic acid and chondroitin sulphate, may also be present in BALF. It was therefore important to demonstrate that high Mw protein identified in BALF by the lectin candidate, UEA-I, was not hyaluronic acid and/or chondroitin sulphate. This was achieved by the incubation of BALF with enzymes specific for hyaluronic acid and chondroitin sulphate digestion: hyaluronidase and chondroitinase respectively. Loss of UEA-I-positive high Mw protein or the presence of low Mw breakdown products by hyaluronidase and chondroitinase degradation would suggest that the high Mw protein present in BALF, identified by UEA-I, was hyaluronic acid or chondroitin sulphate. SDS-PAGE and Western blot was subsequently repeated for the digested BALF samples. Membranes were then stained with UEA-I and the quantity of UEA-I-positive protein was compared to undigested BALF samples.

Additionally it was important to demonstrate that UEA-I was insensitive to blood components that may also be present in guinea pig BALF. SDS-PAGE and Western blot were repeated for samples of lysed guinea pig blood cells and guinea pig plasma. Subsequent membrane staining with UEA-I could identify possible lectin-positive material in blood components.

7.4.4.1 Plasma and Whole blood lysate samples

1. Guinea pig blood was obtained from a naïve guinea pig by cardiac puncture and immediately held in EDTA-coated tubes to prevent clotting.
2. Blood was centrifuged at 1700g for 15mins.
3. The clear supernatant (plasma) was pipetted into glass tubes.

4. The cell pellet was suspended 1:1 with FACS lysing solution and allowed to stand (10mins).
5. 100µl of concentrated BALF, human mucin standard, plasma sample or whole blood lysate (diluted 10x) was added to 80µl of Novex Tris-Glycine SDS Sample buffer (Invitrogen Ltd, UK) and 20µl of NuPAGE Sample Reducing Agent (Invitrogen Ltd, UK). All samples were boiled for 10mins at 100^{0}C and immediately placed on ice for at least one minute subsequent to loading.
6. SeeBlue® Plus 2 (Invitrogen Ltd, UK) was ready for direct application to the gel.
7. 10µl of SeeBlue® Plus 2 standard and 15µl of test samples were applied to designated wells of an SDS-PAGE gel and ran at constant amperage of 35mA with maximum voltage of 125V for 2hrs.
8. The gel was blotted overnight onto a nitrocellulose membrane and the membrane ultimately stained with UEA-I (for detailed methodology, see Section 7.4.3.4).

7.4.4.2 Enzyme digested BALF samples

1. 1ml of concentrated BALF (10x) was incubated with chondroitinase ABC (0.1units) for 30mins at 37^{0}C.
2. 1ml of concentrated BALF (10x) was incubated with hyaluronidase (10units) for 1hr at 37^{0}C.
3. 1ml of concentrated BALF (10x) was incubated in buffer (1ml) for 1hr at 37^{0}C and used as a control.
4. 100µl of digested and control BALF and human mucin standard was added to 80µl of Novex Tris-Glycine SDS Sample buffer (Invitrogen Ltd, UK) and 20µl of NuPAGE Sample Reducing Agent (Invitrogen Ltd, UK). All samples were heated for 10mins at 100^{0}C and immediately placed on ice for at least one minute subsequent to loading.
5. SeeBlue® Plus 2 (Invitrogen Ltd, UK) was ready for direct application to the gel.

6. 10µl of SeeBlue® Plus 2 standard and 15µl of test samples were applied to designated wells of an SDS-PAGE gel and run at constant amperage of 35mA with maximum voltage of 125V for 2hrs.
7. The gel was then blotted overnight onto a nitrocellulose membrane and the membrane ultimately stained with UEA-I (for detailed methodology see Section 7.4.3.4).

7.4.5 Sandwich Enzyme Linked Lectin Assay (Sandwich ELLA) of guinea pig BALF using UEA-I and horse-radish conjugated UEA-I

The Sandwich ELLA has been developed for the detection of soluble lectin binding substances. The assay exploits the mucin binding capacity of the lectin, UEA-I. Immobilised UEA-I acts as a capture lectin and HRP-conjugated UEA-I as the detection lectin.

1. UEA-I was diluted 1.25µg/ml in coating buffer (2.92g $NaHCO_3$, 1.60g Na_2CO_3 made up to 1L with deionised water, pH 9.5). 100µl was added to each well of a 96-well, flat bottomed, high binding, microtitre ELISA plate (Costar, Cambridge USA) and incubated overnight at 4^0C to allow complete binding.
2. The plate was washed with 200µl of freshly prepared wash buffer (PBS (0.01%) with Tween-20 (0.05%), pH 7.4 and 0.05% gelatine, using an Embla automated cell washer.
3. The plate was dried by inverting and applying pressure against a solid surface.
4. 150µl of blocking buffer (PBS (0.01%) with Tween-20 (1%) was added to each well to block remaining sites for protein binding.
5. The plate was covered and incubated at 37^0C for 1hr.
6. The plate was washed with 200µl wash buffer, using an Embla automated cell washer.

7. The plate was dried by inverting and applying pressure against a solid surface.
8. 100µl of human mucin standard was added to the plate in duplicate at an initial concentration of 48 units/ml (diluted 1/2000 from stock) and was serially diluted in PBS over a total of 9 wells.
9. 100µl of PBS was added in duplicate (x3) to the plate to serve as blank samples.
10. 100µl of test samples were added in duplicate to the remainder of the plate.
11. The plate was covered and incubated at 37^0C for 1hr.
12. The plate was washed with 200µl wash buffer (x4) using an Embla automated cell washer, and dried by inverting and applying pressure against a solid surface.
13. 100µl of Horseradish Peroxidase conjugated UEA-I (UEA1-HRP) lectin (Costar, Cambridge USA) diluted 1.25µg/ml in conjugate buffer, was added to each well.
14. The plate was covered and incubated at 37^0C for 1.5hr.
15. The plate was washed with 200µl wash buffer (x6) using an Embla automated cell washer, and dried by inverting and applying pressure against a solid surface.
16. The substrate solution, o-phenylenediamine dihydrochloride (OPD), was prepared under low light conditions immediately prior to use.
17. 150µl of OPD (0.05%) in Citrate Phosphate buffer (0.15M) pH 5.0 containing hydrogen peroxide (0.05%)) was added to each well and allowed to develop in low light conditions at RT for 20min. The oxidation product of OPD by HRP, 2,3-diaminophenazine, is orange-brown in colour.
18. The reaction was stopped by adding 50µl of H_2SO_4 (4M) to each well.
19. The plate well absorbance was measured at a wavelength of 492nm using a SpectraMax 340PC Microplate Spectrophotometer.

20. In order for the assay performance to be considered acceptable, the average blank value should be <30% of the top standard (48units/ml).

7.5 RESULTS

7.5.1 Histological staining of guinea pig lung paraffin sections

7.5.1.1 AB/PAS staining of guinea pig left lung paraffin sections

AB/PAS staining of guinea pig lung paraffin sections successfully identified goblet cell-associated mucin in the bronchiolar epithelium. The majority of goblet cells stained magenta, indicating neutral mucin content. Additional tissue elements were also identified as PAS-positive: basement membranes stained pink and macrophages stained various shades of magenta. Cartilage stained bright blue (Fig. 7.4b)

7.5.1.2 Optimisation of lectin concentrations for immunoperoxidase staining

The appropriate biotinylated lectin concentrations were determined by microscopic examination of adjacent guinea pig lung paraffin sections stained with one of a range of biotinylated lectin concentrations. The lectin dilution that produced the most intense mucin staining was chosen for further studies. For optimised lectin concentrations see Table 7.2.

Lectin	**Lectin concentration range** (dilution of a 1mg/ml lectin conc.)	**Optimised lectin conc.** (dilution of a 1mg/ml lectin conc.)
UEA-I	1/3000-1/12000	1/9000
DBA	1/4000-1/16000	1/8000
HPA	1/6000-1/24000	1/3000
GMA	1/4000-1/16000	1/2000
TVA	1/3000-1/12000	1/3000
VVL	1/1000-1/4000	1/1000

Table 7.2. Optimal biotinylated lectin concentrations for the immunoperoxidase staining of paraffin sections of guinea pig left lung. Lectin concentration range and optimised lectin concentrations are stated.

7.5.1.3 UEA-I staining of guinea pig left lung paraffin sections

Goblet cells in the bronchiolar epithelium of paraffin sections of guinea pig left lung were stained comparably by both UEA-I (purple/magenta) and AB/PAS (brown). Fortunately, excessive background staining observed following AB/PAS staining, including reactivity of tissue elements and macrophages, was not detected subsequent to UEA-I staining (Fig. 7.4)

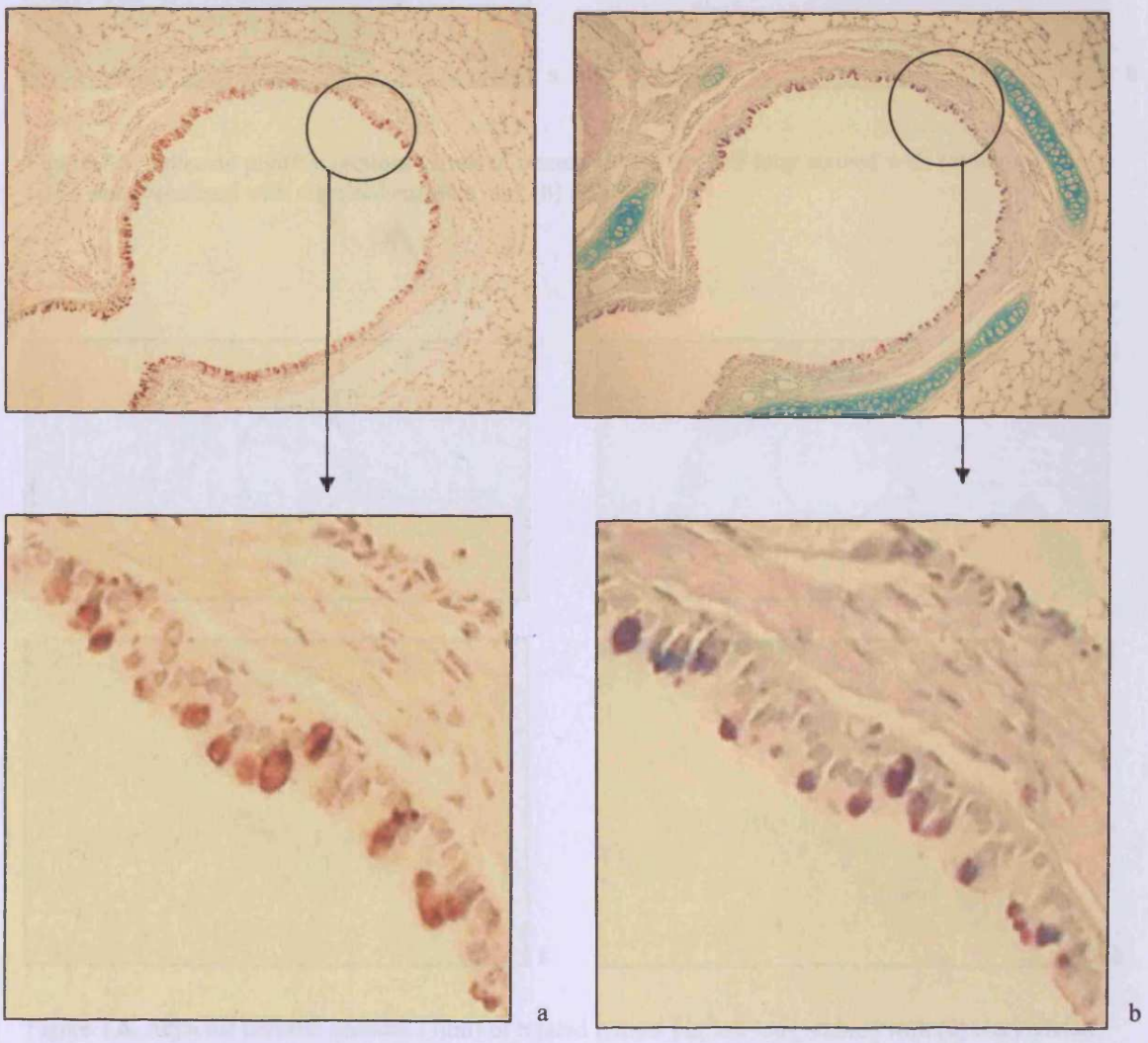


Figure 7.4. Adjacent paraffin sections (3μm) of treated guinea pig left lung stained with (a) biotinylated UEA-I and visualised with diaminobenzidine, and (b) AB/PAS. Magnification of the bronchiolar epithelium in sections (a) and (b) demonstrates goblet cell staining by both UEA-I and AB/PAS respectively.

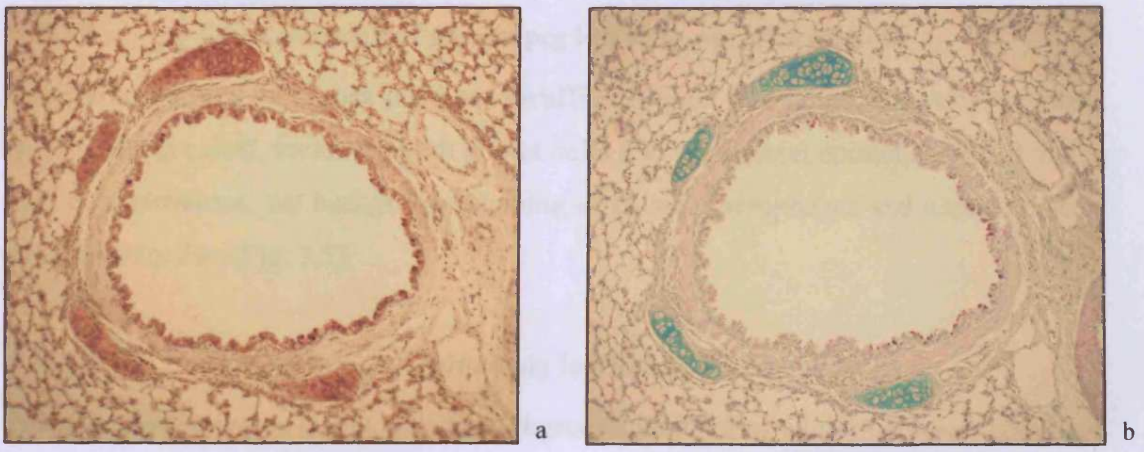


Figure 7.5. Adjacent paraffin sections (3µm) of treated guinea pig left lung stained with (a) biotinylated GMA and visualised with diaminobenzidine, and (b) AB/PAS.

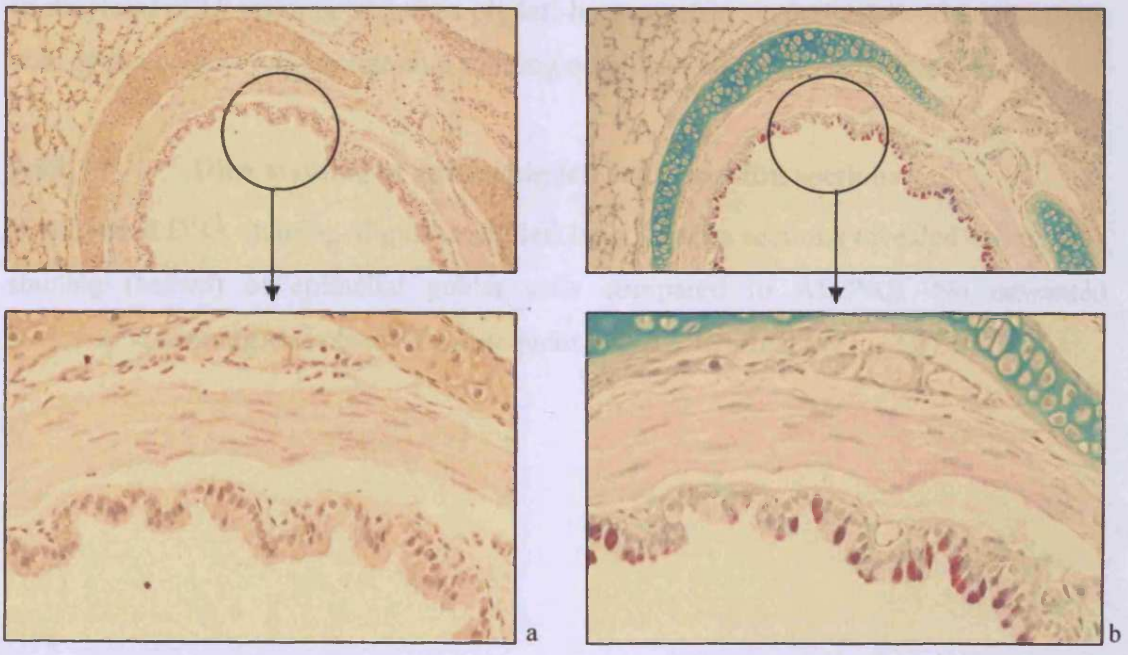


Figure 7.6. Adjacent paraffin sections (3µm) of treated guinea pig left lung stained with (a) biotinylated TVA and visualised with diaminobenzidine, and (b) AB/PAS. Magnification of the bronchiolar epithelium in sections (a) and (b) demonstrates differences in goblet cell staining by TVA and AB/PAS respectively.

7.5.1.4 GMA staining of guinea pig left lung paraffin sections

Excessive staining of guinea pig lung paraffin sections was noted following treatment with GMA (brown), including both goblet cells and non-goblet epithelial cells. Even at low concentrations, the background staining of tissue macrophages and tissue elements was considerable (Fig. 7.5).

7.5.1.5 TVA staining of guinea pig left lung paraffin sections

No selectivity towards goblet cells or background staining of additional tissue structures was observed subsequent to TVA staining, even at high concentrations of TVA (Fig. 7.6).

7.5.1.6 HPA staining of guinea pig left lung paraffin sections

Biotinylated HPA staining of guinea pig left lung paraffin sections showed no reactivity with goblet cells or any background staining of additional tissue structures (Fig. 7.7).

7.5.1.7 DBA staining of guinea pig left lung paraffin sections

Biotinylated DBA staining of guinea pig left lung paraffin sections revealed comparable staining (brown) of epithelial goblet cells compared to AB/PAS. No unwanted background staining was observed subsequent to DBA treatment (Fig. 7.8).

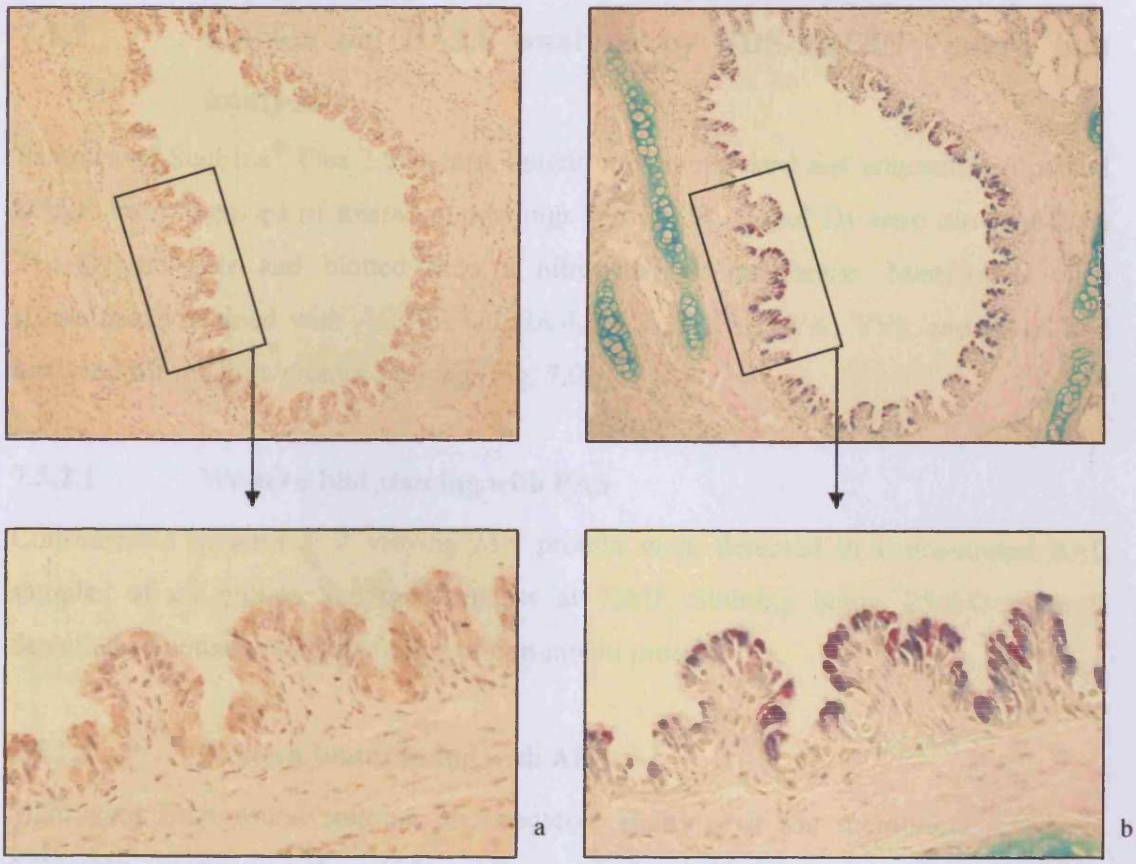


Figure 7.7. Adjacent paraffin sections (3µm) of treated guinea pig left lung stained with (a) biotinylated HPA and visualised with diaminobenzidine, and (b) AB/PAS. Magnification of the bronchiolar epithelium in sections (a) and (b) demonstrates differences in goblet cell staining by HPA and AB/PAS respectively.

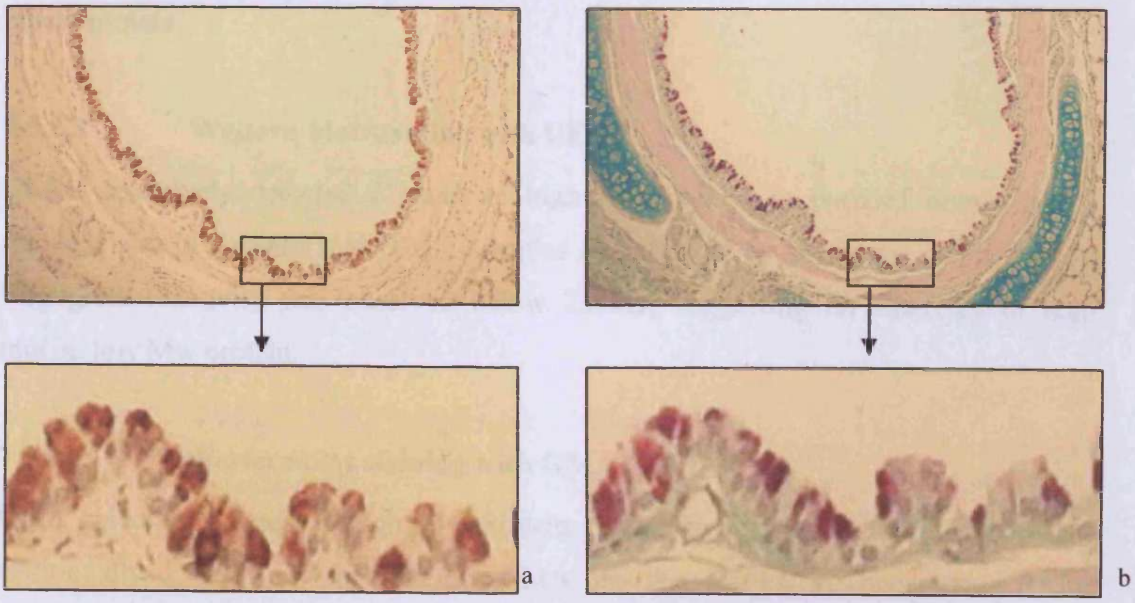


Figure 7.8. Adjacent paraffin sections (3µm) of treated guinea pig left lung stained with (a) biotinylated DBA and visualised with diaminobenzidine, and (b) AB/PAS. Magnified images of the bronchiolar epithelium of sections (a) and (b) demonstrates goblet cell staining by DBA and AB/PAS respectively.

7.5.2 Guinea pig BALF analysis by SDS-PAGE/Western blot analysis

Samples of SeeBlue® Plus 2 standard, human mucin standard and concentrated pooled BALF from 3 groups of treated guinea pigs (groups B, C and D) were run on 4-20% Tris-glycine gels and blotted onto a nitrocellulose membrane. Membranes were subsequently stained with AB, PAS, UEA-I, HPA, DBA, TVA, VVL and GMA and analysed for positive protein staining (Fig. 7.9).

7.5.2.1 Western blot staining with PAS

Considerable quantities of varying Mw protein were detected in concentrated BAL samples of all groups, the most intense at 50kD. Staining below 250kD suggests detection of considerable quantities of non-mucin protein.

7.5.2.2 Western blot staining with AB

Significant background staining and negative staining of the membrane results in difficultly interpreting the results. AB negatively stained considerable amounts of protein of varying Mw in concentrated BALF samples of all groups, the most intense at 50kD. Staining below 250kD suggests detection of considerable quantities of non-mucin protein.

7.5.2.3 Western blot staining with UEA-I

UEA-I selectively detected 2 bands of high Mw protein in purified human mucin standard and in concentrated BALF samples as indicated by the arrow in Fig. 7.9(3). Negligible reactivity was observed below 250kD, suggesting no detection of non-mucin, low Mw protein.

7.5.2.4 Western blot staining with GMA

GMA selectively detected high Mw protein in purified human mucin standard, but additionally detected several proteins of Mw less then 250kD in concentrated BALF samples, particularly significant in the Mw regions 10 and 50kD.

7.5.2.5 Western blot staining with DBA

DBA detected considerable protein ranging from 250kD to 10kD, suggesting reactivity with non-mucin products in concentrated guinea pig BALF. Human mucin standard was not detected by DBA.

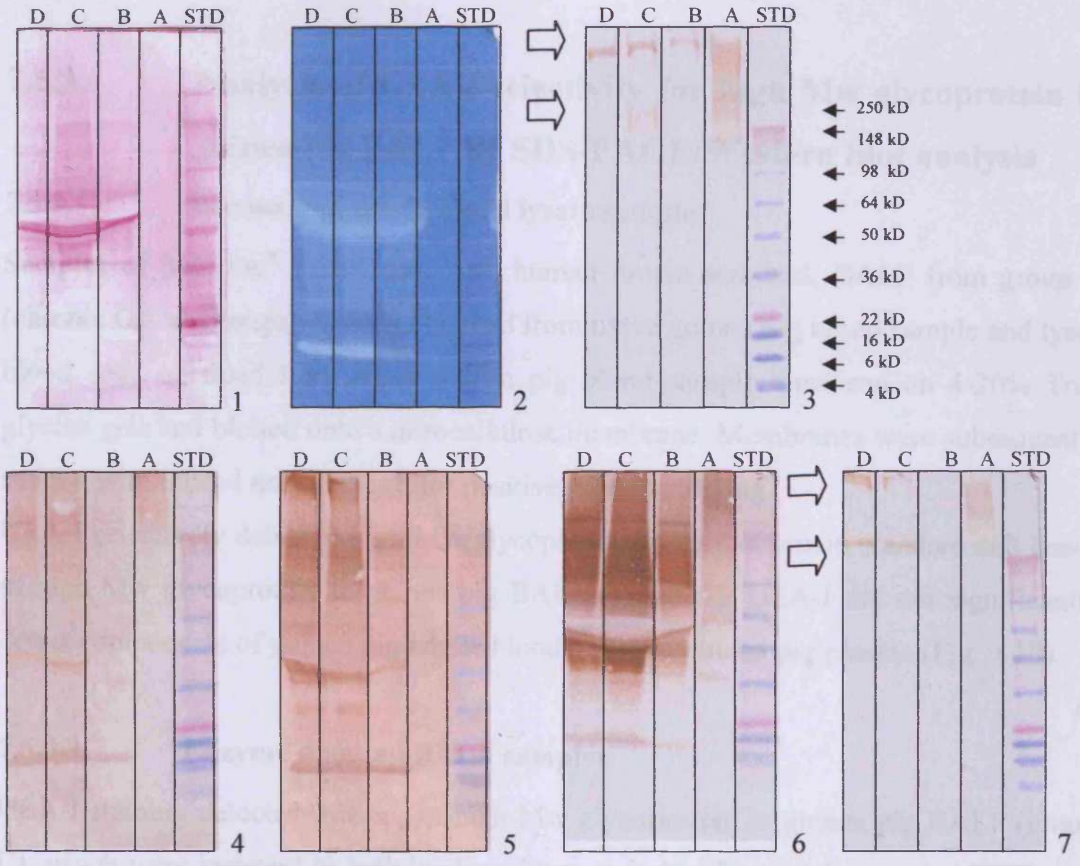


Figure 7.9. Western blots of human standard (A), SeeBlue®Plus2 Pre-Stained Standard (STD), concentrated (10x) BALF removed from acutely challenged guinea pigs (B), concentrated (10x) BALF removed from chronically challenged guinea pigs (C) and concentrated (10x) BALF removed from secretogogue challenged guinea pigs (D), ran on 4-20% Tris-glycine gels and subsequently treated with one of the following stains: 1) PAS, 2) AB, 3) UEA-I, 4) GMA, 5) DBA, 6) TVA and 7) HPA.

7.5.2.6 Western blot staining with TVA

TVA detected considerable amounts of proteins ranging from >250kD to ~10kD, suggesting reactivity to both high Mw protein and non-mucin products in concentrated guinea pig BALF. A large intense protein band was detected in human mucin standard.

7.5.2.7 Western blot staining with HPA

HPA selectively detected 2 bands of high Mw protein in purified human mucin standard and in concentrated BALF samples, as indicated by the arrows in Fig. 7.9(7). Negligible reactivity was observed below 250kD, suggesting insensitivity to low Mw, non-mucin protein.

7.5.3 Analysis of UEA-I-selectivity for high Mw glycoprotein in guinea pig BALF by SDS-PAGE/Western blot analysis

7.5.3.1 Plasma and whole blood lysate samples

Samples of SeeBlue® Plus 2 standard, human mucin standard, BALF from group C (chronic OA challenge), plasma obtained from naïve guinea pig blood sample and lysed blood cells obtained from naïve guinea pig blood sample were run on 4-20% Tris-glycine gels and blotted onto a nitrocellulose membrane. Membranes were subsequently stained with UEA-I and analysed for positive protein staining.

UEA-I selectively detected high Mw glycoprotein in human mucin standard and bands of high Mw glycoprotein in guinea pig BALF (group C). UEA-I did not significantly detect components of guinea pig whole blood lysate or guinea pig plasma (Fig. 7.10).

7.5.3.2 Enzyme digested BALF samples

UEA-I staining detected 2 bands of high Mw glycoprotein in guinea pig BALF (group C), which were resistant to both hyaluronidase and chondroitinase digestion (CHD and HYL) (Fig. 7.11) and revealed no lower Mw digestion products.

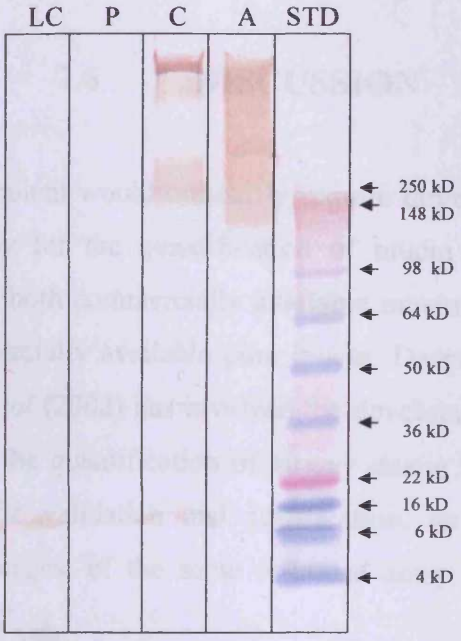


Figure 7.10. Western blots of SeeBlue®Plus2 Pre-Stained Standard (STD), human mucin standard (A), concentrated (10x) BALF removed from chronically challenged guinea pigs (C), guinea pig plasma (P) and diluted (5x) guinea pig lysed blood cells (LC) ran on 4-20% Tris-glycine gels and subsequently stained with UEA-I.

Figure 7.11. Western blots of SeeBlue®Plus2 Pre-Stained Standard (STD), human mucin standard (A), concentrated (10x) BALF removed from chronically challenged guinea pigs (C), concentrated guinea pig BALF, previously digested with hyaluronidase (HYL) and concentrated guinea pig BALF, previously digested with chondroitinase ABC (CHD), ran on 4-20% Tris-glycine gels and subsequently stained with UEA-I.

7.6 DISCUSSION

Successful assay development would ordinarily require target specific antibodies. The development of an assay for the quantification of mucin in biological samples is hampered by the lack of both commercially available mucin specific antibodies and a reliable supply of commercially available pure mucin. Despite this, previous research carried out by Jackson *et al* (2002) has involved the development of an enzyme-linked lectin assay (ELLA) for the quantification of airway mucin in rat biological samples. This chapter detailed the validation and justification, as well as discussing the advantages and disadvantages, of the same in-house assay for the quantification of mucin in guinea pig BALF.

Mucins are threadlike, high Mw glycoproteins, which are polydisperse in size (from 3 to 32 million Dalton) and length (0.5μm-10μm) (Henke *et al* 2004, Thornton *et al* 1990). They consists of a backbone of glycoprotein subunits linked by disulphide bonds (Rogers 1994). Hydroxyl moieties of serine and threonine on the mucin backbone provide sites for o-glycosidic linkages to numerous oligosaccharides, which themselves are composed of multiple combinations of five different sugar types: N-acetylgalactosamine, N-acetylglucosamine, galactose, sialic acid and fucose (Van den Steen *et al* 1998). These sugars are potential targets for specific lectin binding (Fig. 7.1).

Mucins are stored in condensed form in the goblet cells of the airway epithelium. The distinct goblet shaped morphology and specific location of the goblet cell allows the use of the unspecific stain AB/PAS to identify goblet cell-associated mucin in histological sections of lung. This investigation has examined a panel of lectins that have specificities of binding to α-L-fucose, N-acetylglucosamine or N-acetylgalactosamine (Table 7.1) to determine their selectivity for goblet cell mucin. Paired, adjacent guinea pig left lung paraffin sections were stained with either a panel of lectins using the 2-stage avidin-biotin peroxidase technique or AB/PAS. Total mucin content in paraffin sections of guinea pig lung was assessed following AB/PAS staining and compared to the lectin binding profile of adjacent sections. Goblet cell-selective lectins were potential candidates to use in a lectin-based assay to measure mucin. Of the lectins

tested, only UEA-I (which binds α-linked fucose residues) and DBA (which binds α-linked N-acetylgalactosamine residues) demonstrated goblet cell-selective staining comparable to that of AB/PAS. All other lectins examined demonstrated either a lack of reactivity to all airway components in paraffin lung sections or revealed reactivity with additional airway tissue components such as non-goblet epithelial cells, cartilage, inflammatory cells or extracellular matrix.

AB/PAS comparable goblet cell binding by the lectins UEA-I and DBA, in association with minimal background staining, suggests mucin selectivity. A mucin specific lectin binding profile is required to prevent interference of mucin detection by other protein components in biological samples. However, prior to staining with either AB/PAS or lectin, guinea pig lung sections undergo histological processing. This involves multiple treatment stages including formaldehyde fixation, deparaffination in xylene, treatment with graded industrial methylated spirit concentrations and immersion in wax. Extensive processing of tissue samples can lead to altered protein structure and may result in modified reactivity of airway components to different stains. Furthermore, goblet cells consist of mucin and non-mucin components and therefore lectin binding to goblet cells in paraffin sections does not unconditionally equate to a mucin specific lectin.

The Mw of intact mucins can be up to 7000kDa and mucin units are no less than 300 kDa (Rogers 1994, Jackson *et al* 2002). This characteristic allows mucins to be distinguished from other proteins present in BALF. SDS-PAGE and Western blot analysis identified the presence of two bands of high Mw material in guinea pig BALF, subsequent to UEA-I and HPA staining. Other lectins demonstrated significant reactivity with both high and low Mw material in BALF, suggesting reactivity with non-mucin, low Mw protein and little selectivity towards mucin protein. Surprisingly, the reactivity of UEA-I and HPA to high Mw protein in BALF appeared more sensitive than AB or PAS.

SDS-PAGE and Western blot analysis of BALF from rats revealed one distinct band of UEA-I-reactive, high Mw glycoprotein (Jackson *et al* 2002), whereas analysis of guinea pig BALF in this study has identified two distinct high Mw bands following both HPA and UEA-I analysis. Several explanations for this can be hypothesised based on an

understanding of mucin structure and formation. The possible degradation of mucins into different sized subunits may potentially result in multiple Mw protein units in BALF. Secondly, the production of more than one mucin type from respiratory goblet cells, encoded by different MUC genes, may result in the presence of two distinct mucin populations in the airways. Furthermore, two main groups of mucins exist, the oligomeric secretory mucins and the non-oligomeric membrane-bound mucins. Disruption of the bronchiolar epithelium following BAL may stimulate the release of membrane bound mucins from the airway epithelium into airway secretions and BALF.

UEA-I was the only lectin demonstrating both specificity with goblet cells of guinea pig lung paraffin sections and reactivity with high Mw material in guinea pig BALF. It was therefore identified as a suitable candidate for use in a lectin-based assay to quantify BALF mucin. However, further validation was required to demonstrate selectivity of UEA-I to mucin. Two major classes of high Mw glycoconjugates, mucin glycoproteins and proteoglycans, can be secreted from airway epithelial cells (Berger *et al* 1999). Proteoglycans include the glycosaminoglycans, chondroitin sulphate and hyaluronic acid. Chondroitin sulphate provides support in blood vessels, bone, skin and cartilage, while hyaluronic acid is present in the tissue space acting as a binding, lubricating and protecting agent (Schu and Ulsamer 1980). Both proteins have been identified in airway secretions (Schu and Ulsamer 1980) and an increase in hyaluronic acid levels has been demonstrated in the BALF of asthmatic patients compared to healthy individuals (Vignola *et al* 1998). Unlike mucin glycoproteins, hyaluronic acid and chondroitin sulphate are degraded by their respective enzymes, hyaluronidase and chondroitinase. Therefore, in order to identify either proteoglycan following UEA-I staining, BALF was incubated with either hyaluronidase or chondroitinase ABC prior to SDS-PAGE and Western blot analysis. BALF UEA-I reactive high Mw material was hyaluronidase and chondroitinase resistant, suggesting one of two things: 1) UEA-I demonstrates no reactivity to sugar residues of hyaluronic acid and chondroitin sulphate or 2) hyaluronic acid and chondroitin sulphate were absent from the BALF of treated guinea pigs.

Oedema and haemorrhage are inflammatory responses that may occur following the stimulation of an allergic response in guinea pigs, particularly after a chronic response. Although cells, including inflammatory cells and blood cells, are removed from BALF

by centrifugation, it is important to demonstrate insensitivity of UEA-I to blood components such as plasma proteins and blood cells. UEA-I did not react with plasma or blood cell lysate taken from naïve guinea pigs. Therefore the interference of mucin quantification, using the UEA-I-based ELLA, by blood components is minimal.

Detailed optimisation protocols and discussion of the advantages of the UEA-I assay were provided in detail by Jackson *et al* (2002). The assay requires minimal sample preparation and requires no dilution of BALF samples. The assay was previously optimised for primary UEA-I concentration, secondary UEA-I concentration, reaction temperature and incubation times. Inter- and intra-assay variabilities were calculated using varying concentrations of human mucin standard. However, in addition to realising the benefits, it is always important to appreciate the limitations of any assay. The following list highlights some of the main questions raised during the validation of the UEA-I/UEA-I ELLA for the quantification of mucin in guinea pig BALF:

a) It was previously revealed that BALF UEA-I-reactive material was resistant to both hyaluronidase and chondroitinase degradation. Blood components were also found to be insensitive to UEA-I staining. However, it is important to appreciate possible interference of mucin quantification by additional components present in BALF.
b) BALF used in this assay is untreated except for the removal of cells by centrifugation. This process is utilised for the removal and minimisation of cellular contaminants in BALF. However, mucus can be extremely viscous and a proportion of mucus from BALF may be removed by centrifugation.
c) The BAL procedure may result in the disruption of epithelial goblet cells, stimulating degranulation of goblet cells and subsequent mucin release. This may elevate the levels of BALF mucin following pretreatment of the animal. Additionally, the BAL procedure may force mucus into the smaller airways from where it may be more difficult to remove during BAL, thus resulting in decreased levels of mucin in BALF.
d) A significant disadvantage of this assay is the use of an in-house mucin standard rather than a commercially available mucin standard. Further investigation into the use of the Sigma bovine gastric mucin as a standard in the UEA-I/UEA-I ELISA may facilitate development of a more universal assay system.

e) UEA-I does not react with the peptide backbone of mucins, but rather the oligosaccharide component fucose. Therefore, a quantifiable increase in UEA-I reactive material in BALF may be a result of either increased gross BALF mucin content or an increase in oligosaccharide units/fucose sugars and thus lectin reactive sites. The proportion of oligosaccharide units, and therefore fucose sugars, can increase in certain disease states. This can have significant biological effects. Mucin containing a high proportion of fucose is more viscoelastic than mucin with low fucose content (Majima *et al* 1999), resulting in the formation of mucus plugs that are difficult to remove by mucociliary clearance. Furthermore, oligosaccharide sugars permit linkages to bacterial adhesion molecules which may contribute to airway infection and subsequent inflammation (Jefcoat *et al* 2001). Therefore, measurement of increased mucin fucose content, in addition to quantification of increased gross mucus airway content, may be biologically relevant.

f) Commercially available mucin antibodies have not been well characterised and specificity for guinea pig mucin is questionable. Two antibodies, 17Q2 and 45M1, generated against purified rhesus monkey tracheal mucin and human ovarian cyst mucin preparations respectively, have been characterised against mucin for human, rat, pig, and mouse. Further investigation into the reactivity of anti-mucin antibodies with UEA-I reactive high Mw glycoprotein may provide further information about the selectivity of these enzymes for guinea pig mucin.

This study has revealed that UEA-I positive, high Mw protein present in guinea pig BALF was neither hyaluronic acid nor chondroitin sulphate. Additionally, UEA-I does not react with any components of guinea pig blood, including lysed blood cells and plasma. Therefore, the UEA-I Sandwich ELLA, analysed in these studies, can be utilised for the quantification of high Mw protein, likely to be mucin, in both rat (Jackson *et al* 2002) and now guinea pig BALF. This is the first effective assay for the quantification of guinea pig mucin that does not require extensive sample preparation or utilisation of anti-mucin monoclonal antibodies with questionable reactivity with guinea pig mucin.

CHAPTER 8

Analysis of Mucus Content in the BALF of Treated Guinea Pigs.

8.1 INTRODUCTION

In disease, the physiology of the bronchiolar epithelium is altered and the airway secretory potential can be increased via 2 mucus-producing cells: the mucous cells of the submucosal glands and the surface epithelial goblet cells. Diseased respiratory epithelia reveal enlarged submucosal glands (Jackson 2001) and increased number of goblet cells, both in the larger airways (Jeffery 1997) and smaller airways (probably via differentiation of epithelial cells) (Mason *et al* 2005). As a result, diseased airways possess an increased mucin storing capacity, which is maintained by the upregulation of MUC genes during disease and goblet cell growth (Basbaum *et al* 1999).

Allergen exposure in a sensitised guinea pig is a well-established model of human asthma and demonstrates early and late phase asthmatic responses, airway inflammation, and airway hyperreactivity to inhaled histamine (Smith and Broadley 2007). In previous studies we have developed a chronic model of guinea pig asthma, which revealed an accompanying important characteristic of human asthma: increased goblet cell associated stored mucin (Chapter 3). In addition to stimulating mucus production and ultimately epithelial stored mucin (Takeyama 1999), increased mucus secretion has also been demonstrated following nebulised OA exposure in sensitised animals (Agusti *et al* 1998). A multitude of mediators, some of which are released from degranulated, IgE-fixed mast cells following OA exposure, has been implicated in the stimulation of goblet cell and submucosal gland mucin release. To name only a few, these include acetylcholine, VIP, substance P, $P2Y_2$ receptor agonists, histamine, cytokines, tumour necrosis factor-α (TNF-α), platelet activating factor (PAF), prostaglandin, eosinophil cationic protein (ECP), human neutrophil elastase (HNE) and nicotine (see Section 1.1.6). It was therefore of interest to investigate possible mucus secretion, via the quantification of mucin content in BALF, in our established model of chronic asthma.

The mucus secretagogue UTP has been investigated in considerable detail in previous chapters. UTP is a naturally occurring extracellular signalling molecule, which can stimulate goblet cell associated mucus secretion via $P2Y_2$ receptors without initiating an early and late phase asthmatic response (Chapter 4). The effect of nebulised UTP exposure following chronic OA challenge on goblet cell-associated mucin levels was

investigated in Chapter 4. Guinea pigs exposed to nebulised UTP subsequent to chronic OA challenge revealed reduced goblet cell-associated mucin levels in comparison to animals receiving vehicle, suggesting UTP-mediated mucin release from epithelial goblet cells. I.p. administration of the P2 antagonist suramin, 30mins prior to UTP exposure, inhibited the secretagogue-associated reduction in goblet cell stored mucin (Section 4.5.1.6).

The measurement of mucin present in BALF of secretagogue-treated guinea pigs may provide further corroborative evidence on altered mucus output following secretagogue exposure. The UEA-I assay was validated in Chapter 8 for the measurement of high Mw glycoprotein, probably mucin, present in guinea pig BALF. Mucins are high molecular weight glycoproteins consisting of oligosaccharides attached to a peptide backbone via o-glycosidic linkages. Each oligosaccharide is composed of various combinations of different sugar types including N-acetylgalactosamine, N-acetylglucosamine, galactose, sialic acid and fucose (Jefcoat *et al* 2001). The UEA-I Enzyme Linked Lectin Assay (ELLA) exploits the fucose binding property of UEA-I for the quantification of mucin in guinea pig BALF.

In this chapter, the levels of mucin concentrations in the BALF of treated guinea pigs was investigated to establish possible variations between acutely OA challenged and chronically OA challenged animals and to identify the effect of subsequent UTP exposure with and without suramin (a P2 antagonist) pretreatment. In correlation with previous findings, these results may determine the effect of nebulised OA challenge and secretagogue exposures on goblet cell stored mucin concentrations, stimulated mucus output and potential airway mucus accumulation.

8.2 AIMS AND OBJECTIVES

HYPOTHESIS. *The BALF of chronically OA challenged guinea pigs, exposed to nebulised secretagogue, contains increased amounts of mucin due to secretagogue-induced goblet cell degranulation and mucus secretion.*

8.2.1 Aim

The aim of this chapter was to utilise the UEA-I Sandwich Enzyme Linked Lectin Assay (Sandwich ELLA) (validated in Chapter 7) and UEA-I-stained dot blots of BALF for the measurement of mucin in guinea pig BALF in order to demonstrate possible alterations in mucus output in treated guinea pigs.

8.2.2 Objectives

- To establish a reproducible standard curve of known concentrations of human mucin standard using the UEA-I Sandwich ELLA and to utilise the assay for quantification of mucin content in guinea pig BALF.

- To identify changes in BALF mucin concentrations in sensitised guinea pigs exposed to a chronic OA challenge, compared to sensitised guinea pigs exposed to an acute OA challenge.

- To determine whether secretagogue exposure subsequent to chronic OA challenge would increase BALF mucin concentrations in sensitised guinea pigs.

- To demonstrate the effect of a secretagogue antagonist on possible changes in BALF mucin concentrations subsequent to chronic OA challenges and secretagogue exposures.

- To compare quantification of BALF mucin using either the UEA-I Sandwich ELLA method or UEA-I-stained dot blot method. Further, alternative lectins were examined as possible candidates to measure BALF mucin using the dot plot method.

8.3 MATERIALS AND EQUIPMENT

8.3.1 Materials

Human mucus standard was provided by Novartis (Horsham, UK). Purified human mucin was supplied diluted at 1:2000 in 50% glycerol.

All other materials and reagents were supplied by Sigma unless otherwise stated.

8.3.2 Equipment

DeVilbiss Pulmostar Nebuliser (Sunrise Medical Ltd, Wollaston, UK)

Bio-dot apparatus (Bio-Rad Laboratories Ltd, Hercules, USA)

Denley BR401 Centrifuge (Denley, UK)

Hoefer TE Series Transphor EPS (38B12) SDS-PAGE Tank (Hoefer, San Francisco, USA)

Hoefer TE Series Transphor EPS Blotting Unit (Hoefer, San Francisco, USA)

Embla automated cell washer (Molecular Devices, Sunnyvale, USA)

SpectraMax 340PC Microplate Spectrophotometer (Molecular Devices Ltd, Sunnyvale, USA)

8.4 METHODS

8.4.1 Guinea pig treatments

Guinea pigs were sensitised to OA and challenged with one of the following exposure protocols: 1) single exposure of low-dose OA (acutely OA challenged). 2) chronic exposure of high-dose OA (chronically OA challenged). 3) chronic exposure of high-dose OA followed by a nebulised UTP exposure (secretagogue challenged). 4) chronic exposure of high-dose OA followed by a nebulised UTP exposure in the presence of the P2 antagonist suramin (antagonist treated). 5) chronic exposure of saline (chronically vehicle challenged).

8.4.1.1 Sensitisation

All animals were sensitised on days 1 and 5 with an intra-peritoneal (i.p.), bilateral injection of a suspension containing 100μg of OA and 100mg aluminium hydroxide.

8.4.1.2 Acutely OA challenged guinea pigs

14 days following sensitisation (day 15), animals were exposed to a nebulised solution of OA (0.01% for 1hr) in a stainless steel exposure chamber (40cm diameter, 15cm height). A Wright nebuliser was used to supply air at a pressure of 20lb p.s.i and at a rate of 0.3ml/min. If any of the animals looked in distress, they were withdrawn from the chamber and exposure considered complete. 24hrs subsequent to OA exposure guinea pigs were overdosed with an i.p. injection of sodium pentobarbitone (Euthatal 400mg/kg) and the lungs lavaged.

8.4.1.3 Chronically-challenged guinea pigs

14 days following sensitisation (day 15), animals were exposed to a single nebulised solution of low dose OA (0.01% for 1hr), using a Wright nebuliser, in a stainless steel exposure chamber (40cm diameter, 15cm height). Animals were subsequently exposed to a nebulised solution of high dose OA (0.1%) on days 17, 19, 21, 23, 25, 27, and 29. A bilateral intra-peritoneal (i.p.) injection of the H_2 antagonist mepyramine (30mg/kg), 30mins prior to high dose OA exposure on days 17, 19, 21, 23, 25 and 27, protected

against fatal anaphylaxis. 24hrs subsequent to the chronic OA exposure period (day 30), guinea pigs were overdosed with an i.p. injection of sodium pentobarbitone (Euthatal 400mg/kg) and the lungs lavaged.

8.4.1.4 Secretagogue challenged guinea pigs

Animals were chronically challenged with OA as described above. 22hrs 45mins following the chronic OA exposure period, animals were exposed to a single nebulised solution of UTP (10mM) for 15mins. A Wright nebuliser was used to supply air at a pressure of 20lb p.s.i and at a rate of 0.3 ml/min into a sealed Perspex chamber (15 x 15 x 32cm). 1hr subsequent to UTP exposure, guinea pigs were overdosed with an i.p. injection of sodium pentobarbitone (Euthatal 400mg/kg) and lungs lavaged.

8.4.1.5 Antagonist treated guinea pigs

Animals were chronically challenged with OA and subsequently exposed to secretagogue as described above. Suramin (60mg/kg) was dissolved in saline and administered by i.p. bilateral injection 30mins prior to UTP exposure.

8.4.1.6 Chronically vehicle challenged guinea pigs

14 days following sensitisation (day 15), animals were exposed to a nebulised solution of saline (1hr), using a Wright nebuliser, in a stainless steel exposure chamber (40cm diameter, 15cm height) on days 15, 17, 19, 21, 23, 25, 27, and 29. Animals were treated with an intra-peritoneal (i.p.), bilateral injection of the H_2 antagonist mepyramine (30mg/kg), 30mins prior to saline challenge on days 17, 19, 21, 23, 25 and 27. 24hrs subsequent to the chronic vehicle exposure period (day 30), guinea pigs were overdosed with an i.p. injection of sodium pentobarbitone (Euthatal 400mg/kg) and the lungs lavaged.

8.4.2 Bronchoalveolar lavage

Immediately following termination by a lethal overdose of sodium pentobarbitone (Euthatal 400mg/kg), an incision was made in the guinea pig's neck and the trachea was cannulated using a 5cm length of intravenous polypropylene cannula. Saline solution (1ml/100g of animal weight) was instilled into the lungs through the cannula using a

syringe and recovered at 3mins. This procedure was repeated and the recovered lavage fluid combined.

8.4.3 Preparation of guinea pig BALF

Groups of 6 guinea pigs were used. BALF was centrifuged for 6mins at 2000rpm and the supernatant stored at -20°C. BALF was removed from the freezer and allowed to reach room temperature before loading onto the plate.

8.4.4 Sandwich Enzyme Linked Lectin Assay (Sandwich ELLA) of guinea pig BALF using UEA-I

Utilisation of the UEA-I Sandwich ELLA for quantification of mucin in guinea pig BALF was validated in Chapter 7. The assay exploits the mucin binding capacity of the lectin UEA-I. Immobilised UEA-I acts as a capture lectin and HRP-conjugated UEA-I as the detection lectin. Quantification of BALF mucin was measured in the BALF of acutely OA challenged, chronically OA challenged and secretagogue challenged guinea pigs.

8.4.5 Measurement of lectin-positive protein in BALF by Dot Blot analysis.

Further analysis of UEA-I-positive protein in BALF was performed by a dot blot procedure. This allowed comparison of UEA-I-positive BALF protein content to amounts of AB, PAS and other lectin-positive protein in BALF of treated guinea pigs.

8.4.5.1 Preparation of guinea pig BALF

Groups of 6 guinea pigs were used. Immediately following the BAL procedure, BALF was centrifuged for 6mins at 2000rpm and the supernatant stored at -20°C. When required BALF was removed from the freezer and allowed to reach room temperature. 1ml of BALF from each guinea pig were combined and mixed by gentle rotation.

8.4.5.2 Dot Blotting of guinea pig BALF onto a nitrocellulose membrane

1. A trans-blot nitrocellulose membrane (Millipore Co., Bedford UK) was cut to the dimensions of a standard 96 well plate.
2. The membrane was soaked in 100% methanol for 15secs followed by immersion in phosphate buffered saline (PBS) for 2mins.
3. The membrane support plate was placed into position in the vacuum manifold of the Bio-dot apparatus and the sealing gasket positioned above.
4. The pre-soaked membrane was laid directly on top of the sealing gasket in the apparatus.
5. The sample template was positioned on top of the membrane and apparatus sealed tight using 4 screws.
6. A vacuum was applied to the apparatus, ensuring a tight seal.
7. The vacuum was released and 200µl of PBS was added to each well.
8. A vacuum was applied to the apparatus and removed once PBS had drained from each well.
9. Steps 7 and 8 were repeated.
10. 100µl of human mucin standard and test samples were added to designated wells.
11. A vacuum was applied to the apparatus and removed once samples had drained from each well.
12. Steps 7 and 8 were repeated (x2).
13. With the vacuum remaining, the sample template was unscrewed and the nitrocellulose membrane removed and soaked in PBS

8.4.5.3 Membrane staining with HRP-conjugated lectins

14) The membrane was cut into designated sections and each section stained using one of the following staining protocols:

(a) Alcian blue (AB) staining.

(i) The membrane was placed in AB (1%, Surgipath Ltd, Europe) in aqueous acetic acid (3%) for 10mins.

(ii) The membrane was washed extensively in PBS and air-dried.

(b) Periodic acid /Schiff staining.

(i) The membrane was placed in periodic acid (1%) for 10mins.

(ii) The membrane was washed with PBS for 10mins.

(iii)The membrane was placed in Schiff reagent (Surgipath Ltd, UK) for 10mins.

(iv)The membrane was washed extensively in PBS and air-dried.

(c) Lectin staining

(i) The membrane was blocked with BSA (3%) in PBS for 3hrs on a rocking platform

(ii) The membrane was washed with PBS

(iii)The membrane was incubated with one of the following lectins, diluted in BSA (3%), for 2hrs on a rocking platform.

1. Helix pomatia agglutinin (HPA)-HRP conjugated
2. Dolichos bifluros agglutinin (DBA) –HRP conjugated
3. Triticum vulgaris agglutinin (TVA)-HRP conjugated
4. Glycine max agglutinin (GMA)-HRP conjugated
5. Ulex Europaeus-I (UEA-I)-HRP conjugated
6. Vicia villosa lectin (VVL)-HRP conjugated

(iv)The membranes were washed in PBS.

(v) The membranes were added to 3,3'-diaminobenzidine (DAB) solution (0.2mg/ml) in PBS and H_2O_2 (0.01%) for 5mins or until staining was complete.

(vi)The membranes were washed extensively in PBS and air-dried.

The intensity of stain for each sample was then analysed by simple observation.

8.4.6 Statistical analysis

Statistical significance ($p<0.05$) was assessed using ANOVA, followed by Student's t-test or Bonferroni test (mean ± sem, n=6).

8.5 RESULTS

8.5.1 The Sandwich Enzyme Linked Lectin Assay (Sandwich ELLA)

The Sandwich ELLA was utilised to enable measurement of mucin concentrations in BALF of guinea pigs.

8.5.1.1 Standard curve for human mucin

Absorbencies of known concentrations of human mucin standards were read in a Spectramax 340PC Microplate Spectrophotometer (Molecular Devices Ltd., UK) and analysed using a standard curve. The standard calibration curve for human mucin standard was best described using a four parameter fit semi-log curve (Fig. 8.1). The linear portion of the standard curve lay above 15 units/ml of human mucin standard. The relative absorbencies of guinea pig BALF in an UEA-I ELLA could be used to calculate unknown concentrations of mucin in BALF in the linear portion of the standard curve.

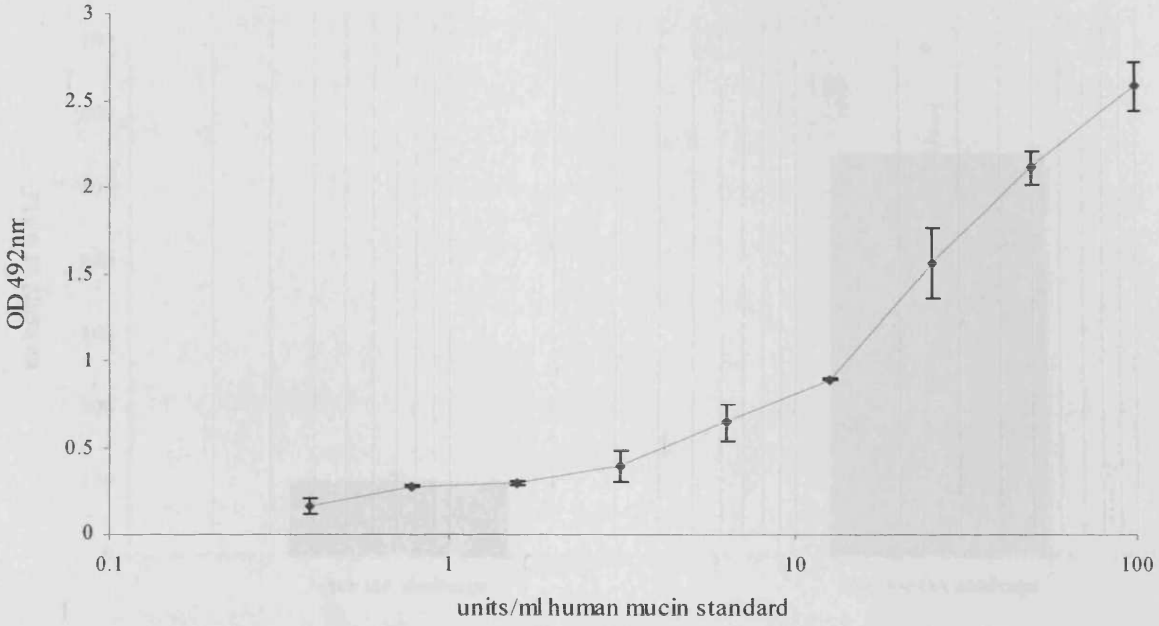


Figure 8.1. Standard calibration curve for human mucin; described using a four parameter fit semi-log curve.
y=((A-D)/1+(X/C)^B))+D.
A=0.198. B=1.187. C=29.58. D=3.135. R^2= 0.998.

8.5.1.2 The effect of chronic OA challenge on BALF mucin content

The units per ml of mucin present in BALF were significantly increased in chronically OA challenged guinea pigs compared to acutely OA challenged animals (Fig. 8.2).

8.5.1.3 The effect of UTP exposures on BALF mucin content in chronically OA challenged guinea pigs

The units per ml of mucin present in BALF were increased significantly in chronically OA challenged guinea pigs compared to animals exposed to a chronic vehicle challenge. A single nebulised exposure to UTP (10mM for 30mins) in animals previously exposed to a chronic OA challenge also revealed a significant increase in the mucin content per ml of BALF, compared to a chronic vehicle challenge. However, this was not significantly different from the group chronically exposed to OA without UTP exposure. Additionally, an i.p. injection of suramin, 30mins prior to UTP exposure had no significant effect on mucin content per ml of BALF, when compared with UTP challenged animals (Fig. 8.3).

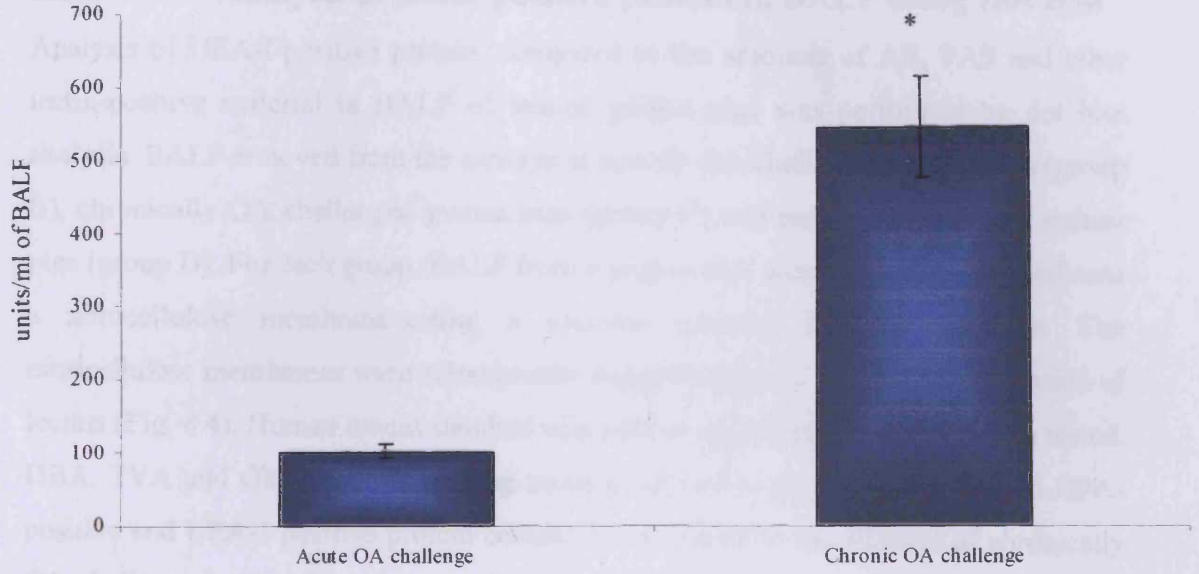


Figure 8.2. The effect of chronic OA challenge compared to acute OA challenge in sensitised guinea pigs on the mucin content (units per ml) of BALF. *($p<0.05$) significantly different from acute OA challenge.

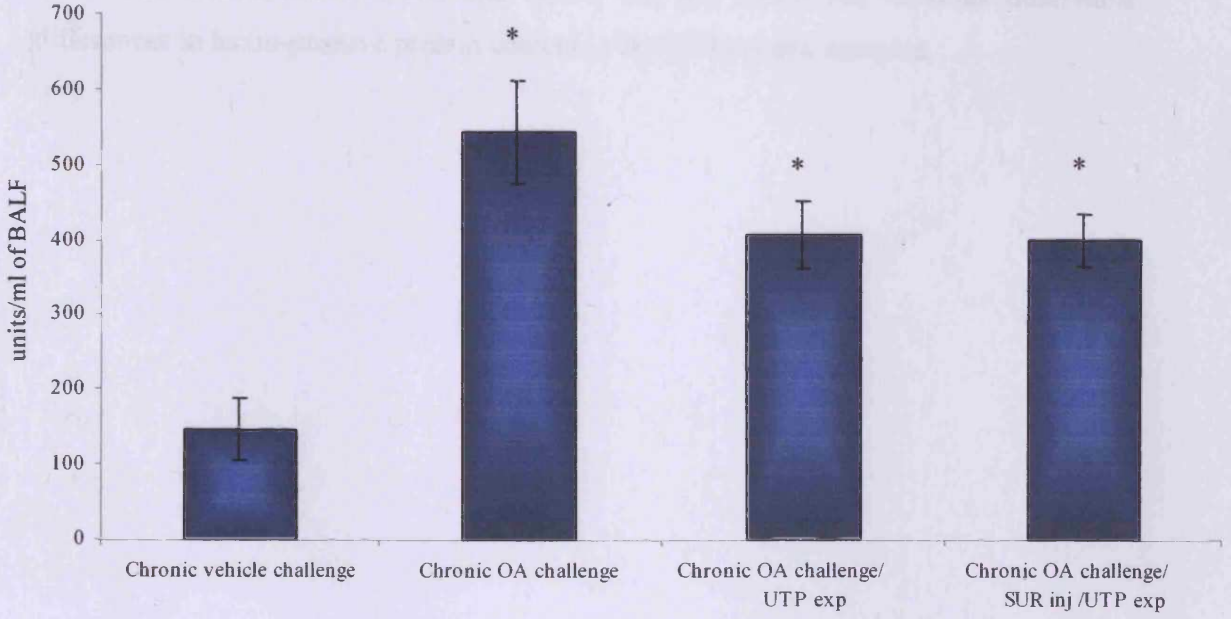


Figure 8.3. Mucin content (units per ml) in the BALF of sensitised guinea pigs challenged with one of the following exposure protocols: chronic vehicle challenge; chronic OA challenge; chronic OA challenge followed by a UTP exposure (10mM/30mins); chronic OA challenge followed by UTP exposure (10mM/30mins), 30mins subsequent to an i.p. injection of suramin (60mg/kg) *($p<0.05$) significantly different from chronic vehicle challenge.

8.5.2 Analysis of lectin-positive protein in BALF using Dot Blot

Analysis of UEA-I-positive protein, compared to the amounts of AB, PAS and other lectin-positive material in BALF of treated guinea pigs was performed by dot blot analysis. BALF removed from the airways of acutely OA challenged guinea pigs (group B), chronically OA challenged guinea pigs (group C) and secretagogue treated guinea pigs (group D). For each group, BALF from 6 guinea pigs were pooled and blotted onto a nitrocellulose membrane using a vacuum operated Bio-dot apparatus. The nitrocellulose membranes were subsequently stained with AB, PAS or one of a panel of lectins (Fig. 8.4). Human mucin standard was positively stained by all the lectins tested. DBA, TVA and GMA revealed strong staining of guinea pig BALF. Increased HPA-positive and UEA-I-positive protein content was revealed in the airways of chronically OA challenged guinea pigs compared to acutely OA challenged animals. Furthermore, increased HPA-positive protein content was revealed in secretagogue treated animals compared to both acutely OA challenged and chronically OA challenged animals. This was also observed following UEA-I staining of guinea pig BALF, but not as strongly as HPA. The lectins, DBA, TVA, and GMA, did not reveal any obvious observable differences in lectin-positive protein content in BALF between samples.

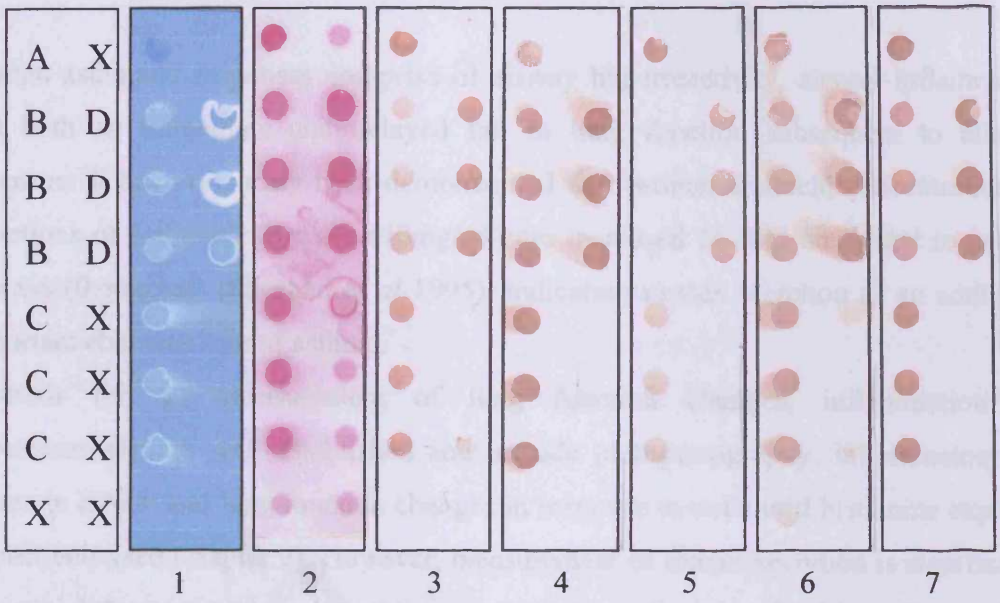


Figure 8.4. Dot blots of human mucin standard (A), BALF removed from acutely challenged guinea pigs (group B), chronically challenged guinea pigs (group C), secretagogue challenged guinea pigs (group D) and blank (X) onto nitrocellulose membranes and subsequently treated with one of the following stains: 1) AB, 2) PAS, 3) UEA-I, 4) DBA, 5) HPA, 6) TV and 7) GM

8.6 DISCUSSION

Human asthmatic responses comprise of airway hyperreactivity, airway inflammation and both an immediate and delayed fall in lung function subsequent to allergen exposure. It has previously been demonstrated that estimated mucin concentrations in secretions of asthmatic airways (40mg/ml) are increased 66-fold compared to healthy airways (0.6mg/ml) (Sheehan *et al* 1995), indicating mucus secretion as an additional important characteristic of asthma.

Methods for the measurement of lung function changes, inflammation and hyperreactivity are well established and include plethysmography, inflammatory cell counts in BALF and lung function changes in response to nebulised histamine exposure respectively (see Chapter 2). However, measurement of mucus secretion is significantly less straightforward and inadequately reported. Quantification of goblet cell-associated mucin content is achieved by histological and morphometric analysis of AB/PAS stained paraffin sections of guinea pig lung. This can provide information regarding stored epithelial mucin content. The UEA-I Sandwich ELLA, developed for the measurement of mucin concentrations in the BALF of treated guinea pigs, was validated and discussed in detail throughout Chapter 7. Quantification of BALF mucin concentrations may provide an informative means to enable analysis of altered mucus output following various exposure protocols and may prove to be a valuable tool for the study of mucus hypersecretion in respiratory disease such as asthma.

A nebulised OA challenge in sensitised animals has been exploited as an asthma model in several experimental animals (Smith and Broadley 2007, Okamoto *et al* 2006). The effect of chronic OA challenges on lung function, inflammatory cell recruitment to the airways, hyperreactivity to inhaled histamine and stored goblet cell-associated mucin in the bronchiolar epithelium was discussed in detail in Chapter 3. A chronic high dose OA challenge in sensitised guinea pigs produced a significant increase in stored goblet cell-associated mucin, compared to an acute OA challenge or chronic vehicle challenge (Chapter 3), demonstrating OA-induced goblet cell-associated mucin production. Analysis of BALF mucin content utilising the UEA-I Sandwich ELLA revealed significantly greater mucin levels in the BALF of guinea pigs exposed to a chronic OA challenge, compared to acutely OA challenged animals. These findings suggest that in

addition to stimulating mucin production and ultimately increased goblet cell associated stored mucin, increased mucin output was also stimulated during a chronic OA challenge. This is consistent with work by Agusti *et al* (1998), who proposed goblet cell mucus secretion following allergen exposure in sensitised animals.

The mucus secretagogue, UTP has been shown to stimulate increased mucus secretion in SPOC1 cells derived from rat tracheal epithelium (Rossi *et al* 2004), human bronchial epithelial cells (Chen *et al* 2001) and canine tracheal epithelial explants (Davis *et al* 1992). The effect of UTP on the percentage goblet cell-associated mucin in the bronchiolar epithelium was analysed by histological analysis and AB/PAS staining in Chapter 4. In chronically OA challenged guinea pigs a reduction in the percentage of AB/PAS-positive epithelial area was revealed following nebulised UTP exposure (10mM for 30mins), suggesting mucus output from goblet cells. Pretreatment with an i.p. injection of suramin (60mg/kg) 30mins prior to UTP exposure inhibited the UTP-associated reduction in goblet cell associated mucin (Section 4.5.1.6). Furthermore, previous lung function analysis revealed a gradual fall in sG_{aw}, lasting up to 1hr following a single nebulised UTP challenge and that was also inhibited by pretreatment with suramin (Section 4.5.1.3).

The effects of UTP nebulised exposure on lung function responses and epithelial stored mucin were consistent with goblet cell degranulation and resulting mucus secretion. It was therefore proposed that goblet cell mucin secretion may be stimulated by UTP, mediated via P2 receptors. Increased mucin output from goblet cells following secretagogue exposure should have resulted in luminal mucus accumulation and consequently increased BALF mucus content. However, no significant differences in BALF UEA-I-reactive glycoprotein concentrations, measured by the UEA-I Sandwich ELLA, were revealed following UTP exposure in chronically OA challenged guinea pigs compared to chronically OA challenged and non-UTP exposed guinea pigs. Additionally, a single i.p. injection of suramin, a P2 receptor antagonist, 30mins prior to secretagogue exposure revealed no significant differences in BALF UEA-I-reactive glycoprotein concentrations from the level in chronically OA challenged guinea pigs. These unexpected findings do not correspond with previous lung function and histology results, which suggested UTP-mediated mucin secretion via P2 receptors. The following paragraphs aim to address possible explanations for these inconsistent results.

- Mucus clearance

In health, mucus is secreted from goblet cells and the mucous cells of the submucosal glands to protect the airway epithelium from damage by bacteria and foreign particles. Clearance of mucus then follows, facilitated either by beating cilia (which waft the mucus towards the mouth to be swallowed), or by cough (which dislodges mucus from the airways). Effective mucociliary clearance (MCC) is determined by physical properties of mucus, such as its viscosity, elasticity and adhesivity (Mason 2005), which are in turn determined by the proportion of mucin present in the airways, sol volume and mucus quantity (Rogers 1994). Mucus is transported by MCC at a rate of about 10mm/min in the larger airways and about 1mm/min in the smaller airways, resulting in the efficient removal of materials present in the lungs within 24 hours (Godwin 2002). In our model, BAL procedure was performed 1hr subsequent to secretagogue exposure. This time period is unlikely to permit MCC-mediated mucus clearance from the airways. However, an additional mechanism for the effective removal of mucus from the airways is the cough reflex. Coughing or sneezing was observed in sensitised and chronically exposed guinea pigs following nebulised secretagogue exposure. Possible stimulation of the cough reflex by respiratory mucus accumulation may lead to removal of goblet cell-secreted mucus from the airways. It would therefore be interesting to examine BALF mucus content at varying time points subsequent to secretagogue exposure. Optimisation of the time interval between secretagogue exposure and BAL would ensure capture of maximal mucus content in guinea pig BALF and would produce more accurate results.

- Specificity towards mucins

Alternative stimuli may induce the secretion of structurally different mucins, possibly from subsets of goblet cell populations in the airway epithelium. Mucins are expressed in 2 major forms: the membrane tethered mucins, which are found on epithelial cells, and the secreted mucins (Voynow 2002). MUC5AC and MUC5B are the main genes encoding for the mucus-forming mucins and are both expressed in goblet cells. MUC5AC and MUC5B are thought to make up to 97% weight of the gel-forming mucins in airways (Phillips *et al* 1996) but differ in size and structure. The oligosaccharide groups of mucins can be linear or branched and vary in size (Rose 1992). The UEA-I ELLA reacts with and identifies the fucose sugars of mucin

oligosaccharides. Alterations in fucose content or fucose positioning within the oligosaccharide unit of mucins may limit UEA-I reactivity. This may result in imprecise BALF mucin quantification and may be a possible limitation to the use of the UEA-I ELLA for the quantification of mucin in this model.

- Inter-individual differences

The use of the UEA-I ELLA for the quantification of mucin in guinea pig BALF is significantly hindered by the absence of an internal control. Interspecies differences are always a factor and important consideration when utilising animal models for scientific research. Measurement of airway mucin concentrations prior to treatment would be the most accurate and favourable choice of control. However, this model and use of the BAL procedure does not permit the use of an internal control, resulting in possible large variations in results.

- Experimental limitations

In asthmatic airways, mucus plugs occlude both the large and small airways (Jeffery 1992). However, although removal of mucus from large airways is presumable, it is possible that mucus accumulated in the smaller airways cannot be removed. Additionally in disease, alterations in mucus composition can occur. Both increased mucin content and high fucose content can result in increased mucus viscosity (Rogers 1994, Majima *et al* 1999). Pressure from the BAL procedure may result in the propulsion of viscous mucus into the smaller airways where it accumulates and may not be removed in BALF. Furthermore, viscous and ultimately denser mucus may be removed from BALF during the centrifugation process, which is utilised for removal of cellular components from BALF. These factors should be considered during evaluation of BALF mucin content

The quantification of UEA-I positive material using the UEA-I Sandwich ELLA did not correlate with quantification utilising UEA-I-stained dot blots of guinea pig BALF. The UEA-I Sandwich ELLA identified increased BALF mucin concentrations in chronically OA challenged guinea pigs, compared to acutely OA challenged guinea pigs. It did not reveal significant differences in the BALF mucin content of secretagogue-treated guinea pigs, compared to chronically OA challenged guinea pigs. However, dot blot analysis of BALF samples revealed observable increases in UEA-I positive protein content in secretagogue treated, compared to chronically OA challenged guinea pigs and in

chronically OA challenged, compared to acutely OA challenged animals. This suggests that although the UEA-I Sandwich ELLA can detect large increases in BALF mucin, it cannot detect subtle changes.

In dot blot analysis, HPA also revealed an observable increase in HPA-positive material in BALF of secretagogue-treated animals compared to chronically OA challenged animals. The difference in BALF lectin-positive material in secretagogue-treated, compared to chronically OA challenged animals was greater following HPA staining of dot blots, compared to staining with UEA-I. However, UEA-I was the unique lectin that provided evidence for both goblet cell-associated mucin staining in histological sections of guinea pig lung and specific staining of high molecular weight protein in BALF (Chapter 8). It was therefore concluded that UEA-I was the most suitable candidate to incorporate in a Sandwich ELLA for quantification of mucin in guinea pig BALF. Despite demonstrating specific staining of high molecular weight protein in guinea pig BALF, HPA, which identifies oligosaccharide N-acetylgalactosamine sugars, did not reveal goblet cell-associated staining of guinea pig lung histological sections (Chapter 8). However, the organisation of stored mucin in airway goblet cells may prevent the accessibility of N-acetylgalactosamine molecules for HPA binding, and therefore prevent identification of goblet cell associated mucin in histological sections of guinea pig lung by HPA. Further investigation may result in the justification of HPA as a more suitable lectin candidate to quantify BALF mucin content in a Sandwich ELLA rather than UEA-I, although time restrictions prevented this.

In conclusion, this study has identified a significant increase in the concentration of BALF mucin in chronically OA challenged guinea pigs compared to acutely OA challenged or vehicle exposed guinea pigs. We have therefore demonstrated not only an increase in goblet cell mucin production following chronic OA challenge, but also increased mucus output. These findings have provided further corroborative evidence supporting the use of chronic OA challenge as a useful model of mucus hypersecretion. However, although previous investigations have provided evidence for $P2Y_2$-stimulated mucus secretion, and associated reductions in lung function, this was not consolidated by quantification of BALF mucin content.

CHAPTER 9

General discussion

9.1 GENERAL DISCUSSION

9.1.1 MAIN AIMS AND METHODS

Several inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) are all characterised by mucus hypersecretion, which can contribute to airway obstruction and deterioration of health (Jackson *et al* 2001). All stages of human asthma, from mild to severe, have been associated with proportional increases in goblet cell populations and stored goblet cell-associated mucin (Ordonez *et al* 2001). Additionally, mucus secretion can be increased in the asthmatic airway. Sheehan *et al* (1995) demonstrated a 66-fold increase in mucin concentrations in secretions of asthmatic airways compared to healthy subjects. However, although there has been intense research investigating the mechanisms behind the development of a mucus hypersecretory phenotype, there has been little research into the potential effect of mucus secretion on lung function. The objective of this project was to identify potential changes in lung function responses following airway challenges in an animal model displaying a mucus hypersecretory phenotype. Experimental protocols, both previously established and newly developed in the laboratory were utilised to achieve this aim. The guinea pig model of acute asthma was previously optimised and has been extensively used in this laboratory (Toward and Broadley 2004, Smith and Broadley 2007). Consistent with findings by Smith and Broadley (2007), the present studies have demonstrated an allergic response following a single nebulised ovalbumin (OA) challenge in OA sensitised guinea pigs. The response was characterised by early and late phase bronchoconstriction (EAR and LAR) (measured as reductions in specific airway conductance (sG_{aw})), airway hyperreactivity (AHR) to inhaled histamine and inflammation (measured as an increase in bronchoalveolar lavage (BAL) inflammatory cell numbers); all features of human asthma (Fig. 3.1, 3.2 and 3.3 respectively). However, increased mucus production and secretion are additional important features of human asthma and it was therefore essential to measure both in the guinea pig model of asthma.

Guinea pig goblet cell-associated mucin production in the airways was quantified by histological and morphometric analysis of alcian blue/periodic acid schiff (AB/PAS)-

stained paraffin sections of guinea pig left lung. AB and PAS are non-specific stains that react with general proteins found in many cell types and tissue components. However, airway goblet cells have a distinct morphology (goblet-shaped) and specific location within the epithelial layer. As a result, AB and PAS are routinely used for the positive identification of mucin in airway histological sections of several laboratory animals (Sueyashi *et al* 2004, Komori *et al* 2001). Quantification of mucin production within the airway epithelium was achieved by analysis of AB/PAS-stained paraffin sections of guinea pig left lung by a SigmaScan Image Analysis program (Systat Software Inc., London, UK). The area of AB/PAS-positive epithelial cells was expressed as a % of the total epithelial area.

In the guinea pig model of asthma, acutely OA challenged guinea pigs revealed no significant difference in the amount of epithelial stored mucin compared to vehicle challenged animals (Fig. 3.5). However, clinically asthma is described as a chronic disease resulting form repeated exposure to inhaled allergen. Although the acute OA model of asthma has been extensively investigated, there has been significantly less focus on airway responses following chronic allergen challenges. For these reasons, a model of chronic asthma was developed. The established model of chronic asthma consisted of one low dose OA exposure and seven consecutive high dose OA exposures, all but the ultimate exposure preceded by an i.p. injection of mepyramine to protect against fatal anaphylaxis (chronic OA challenge 4, Chapter 3). The ultimate high dose OA exposure revealed all human asthma responses that were demonstrated following acute challenge, including EAR, LAR, AHR and inflammatory cell infiltration into the airways (Figs. 3.18, 3.19 and 3.20). Histological analysis also revealed significant goblet cell-associated mucin accumulation, demonstrating increased mucin production, in chronically OA challenged guinea pigs compared to acutely OA challenged or vehicle challenged guinea pigs (Fig. 3.21).

Few scientific papers have detailed adequate protocols for the measurement of mucin in biological samples. However, Jackson *et al* (2002) previously described an enzyme-linked lectin assay (ELLA) that allows measurement of airway mucin in rat biological samples. The ELLA exploited the lectin UEA-1, which reacts with core and terminal α-linked fucose residues on mucin oligosaccharides. Validation of the same ELLA to

quantify guinea pig BALF mucin was described in Chapter 7. Analysis of BALF mucin content revealed significantly greater mucin levels in the BALF of guinea pigs exposed to a chronic OA challenge, compared to acutely OA challenged animals (Fig. 8.2), suggesting that as well as an increase in mucin production, mucin output was stimulated during a chronic OA challenge. This is consistent with studies by Agusti *et al* (1998), who measured AB/PAS stained mucosubstances in the epithelium of sensitised guinea pigs before and subsequent to OA exposure using a semiautomatic imaging system. Application of OA to the trachea of OA-sensitised guinea pigs resulted in goblet cell degranulation and release of epithelial-stored mucosubstances.

In summary, a chronic OA challenge was optimised in guinea pigs to reveal increased goblet cell-associated mucin accumulation (measured as the % of AB/PAS-positive epithelial area) (Chapter 3) and increased mucin secretion (measured as increased BALF UEA-1-positive protein) (Chapter 9), as well as EAR, LAR, AHR, and BALF inflammatory cell influx.

9.1.2 TOLERANCE

Optimisation of the guinea pig model of chronic asthma introduced the phenomenon of respiratory tolerance into the present studies. It is widely accepted that moderation of clinical allergy can be achieved through repeated allergen exposure (Platts-Mills *et al* 2003) and studies by Schramm *et al* (2004) have revealed unresponsiveness to repeated low dose OA challenge in a mouse model of asthma. In the present studies, the development of tolerance to OA was a consequence of repeated exposures to low dose OA in the sensitised guinea pig (Fig. 3.10 and 3.12). The exact mechanisms behind the development of tolerance are unclear, despite considerable research. Possible mechanisms of respiratory tolerance were discussed in some detail in Chapter 3, but were not investigated further as they were not the main objective of the study. These include 1.) clonal deletion (apoptosis and elimination of memory T cells), 2.) anergy (inactivation of T cells), 3.) immune deviation from a Th2 to a Th1 immune response (possibly by γδ CD8+ T cells via the secretion of inhibitory cytokines such as IFN-γ) (McMenamin and Holt 1993) and 4.) active suppression of immune response (possibly

by regulatory T cells (T_{reg}), via the secretion of regulatory cytokines such as IL-10, IL-4 and TGF-β).

However, tolerance observed in the chronic low dose OA model of asthma revealed split tolerance with loss of some inflammatory responses, such as EAR, LAR and AHR but not eosinophilia (Fig. 3.13) or goblet cell mucin accumulation (Fig. 3.21). Additionally, responsiveness to inhaled OA (demonstrated as EAR, LAR, AHR and increased inflammatory cell numbers) was also reinstated to that observed following acute OA challenge, by a 10fold increase in OA dose (Figs. 3.15, 3.16 and 3.17 respectively). Therefore, development of tolerance in the guinea pig model of chronic asthma may be a result of overlapping and linked mechanisms mediated by several cell types and secreted cytokines, rather than due to one distinct pathway.

9.1.3 SECRETAGOGUES

The guinea pig model of chronic asthma, developed in Chapter 3 consisted of 1 low-dose OA challenge, followed by 7 high-dose OA challenges, all except the ultimate challenge being preceded by an i.p. injection of mepyramine maleate (30mg/kg) 30mins prior to OA challenge. As discussed in chapters 3 and 4, in the guinea pig model of asthma, chronic OA challenge induced significant epithelial stored mucin accumulation, measured by analysis of AB/PAS-stained bronchiolar epithelial area. This increased capacity to store goblet cell-associated mucin and increased potential to secrete large amounts of mucus provided a valuable tool to study the effect of mucus secretagogues on goblet cell-stored mucin and airway inflammatory responses, such as mucin secretion and lung function responses.

Mucin is stored in association with high concentrations of neutralising calcium ions within intracellular granules of goblet cells (Rogers 1994). Activation of goblet cell-surface receptors results in activation of various ion channels, leading to a Ca^{2+}/K^{+} exchange and ultimately mobilisation of intracellular Ca^{2+} stores. Subsequent osmotic movement of water into the granule results in hydrolysis and expansion of mucin and the exocytotic secretion of mucin from goblet cells into the airway lumen. Goblet cell-associated mucin secretion can be induced via secretagogue-mediated activation of goblet cell surface receptors. In diseased airways, numerous substances and multiple

receptor subtypes appear to be involved in goblet cell mucus secretion. Five potential secretagogues were used in the present studies, UTP, ATP, UDP, 5'AMP (adenosine) and histamine, which activate the $P2Y_2$ receptor (Chen *et al* 2001), the A_3 receptor (Young 2006) and the H_2 receptor (Tamaoki *et al* 1997) respectively to stimulate goblet cell degranulation. A brief description of the known pharmacology of these secretagogues will precede a discussion of the results obtained in the guinea pig model.

9.1.3.1 $P2Y_2$ receptor-mediated mucin secretion

Goblet cell-associated mucin secretion may be stimulated by activation of the $P2Y_2$ receptor. However, $P2Y_2$ receptor-mediated goblet cell-associated mucin secretion appears to be species-dependent. For example, UTP can stimulate increased mucus secretion in SPOC1 cells derived from rat tracheal epithelium (Rossi *et al* 2004), whilst ATP and UTP are equipotent in stimulating mucin release from hamster epithelial cells (Kim *et al* 1996) and tracheal goblet cells in canine tracheal epithelium (Davis *et al* 1992). However in contrast, work by Roger (2000) revealed that ATP but not UTP could induce MUC5AC mucin secretion from goblet cells in human bronchi. Additionally, there is also evidence that UDP, the metabolite of UTP, may function as a weak agonist at $P2Y_2$ receptors to stimulate goblet cell mucin secretion (Chen *et al* 2001, Choi *et al* 2005).

9.1.3.2 A_3-receptor mediated mucin secretion

There is significantly less information available regarding goblet cell-associated mucin secretion via activation of the adenosine receptors. However, whilst less potent than its triphosphate and diphosphate counterparts (Chen *et al* 2001), adenosine can also stimulate mucus secretion from mouse airway epithelial secretory cells via activation of the A_3 receptor (Young 2006).

9.1.3.3 H_2 receptor mediated mucin secretion

Goblet cell-associated mucin secretion may also be stimulated by activation of the H_2 receptor in the airways. Histamine-induced goblet cell degranulation has been revealed in *in vivo* studies of guinea pig trachea (Tamaoki *et al* 1997). Additionally, goblet cell

mucus secretion may also be stimulated by histamine indirectly, via activation of H_1 receptors on cholinergic nerve terminals and subsequent release of the acetylcholine (ACh) (Takeyama *et al* 1996).

9.1.4 EFFECT OF INHALED SECRETAGOGUE ON MUCIN SECRETION AND LUNG FUNCTION RESPONSES IN THE GUINEA PIG

The effect of inhaled UTP on both lung function responses and goblet cell-associated stored mucin was analysed in Chapter 4. In chronically OA challenged guinea pigs, inhaled UTP (1mM and 10mM for 15mins) revealed a gradual reduction in lung function, reaching a nadir at 45mins and persisting up to 1hr (Fig. 4.8), in addition to a significant reduction in epithelial stored mucin (Fig. 4.9), suggesting mucus output from goblet cells. Pretreatment with an i.p. injection of suramin (60mg/kg) 30mins prior to UTP exposure inhibited both the UTP-induced reduction in sG_{aw} (Fig. 4.5) and the reduction in goblet cell-associated stored mucin (Fig. 4.7). The effects of nebulised UTP exposure on lung function responses and epithelial stored mucin were consistent with goblet cell degranulation and resulting mucus secretion. It was therefore proposed that in chronically OA challenged guinea pig, UTP induced P2 (probably $P2Y_2$)-mediated goblet cell mucin secretion, resulting in mucus accumulation in the airways and a subsequent reduction in lung function.

Increased mucin output from goblet cells following UTP exposure should have resulted in luminal mucus accumulation and consequently increased BALF mucus content. However, no significant differences in BALF UEA-I-reactive glycoprotein concentrations measured by the UEA-I Sandwich ELLA were revealed following UTP exposure in chronically OA challenged guinea pigs (Fig. 8.3). Additionally, a single i.p. injection of the P2 receptor antagonist, suramin, 30mins prior to secretagogue exposure, had no effect on BALF UEA-I-reactive glycoprotein concentrations in chronically OA challenged guinea pigs (Fig. 8.3). These findings were unexpected and will be discussed further later.

A nebulised exposure of a threshold dose of histamine (1mM for 20secs) can be used to demonstrate AHR in acutely OA challenged guinea pigs. Both acutely OA challenged and chronically OA challenged guinea pigs reveal an immediate, but quickly recovered

bronchoconstriction following a threshold dose of nose-only administered histamine (Figs. 3.2 and 6.2). However, this was not associated with a reduction in epithelial stored mucin in chronically OA challenged animals (Fig. 6.6), suggesting no goblet cell-associated mucin secretion. Alternatively, a gradual reduction in lung function (up to 1hr) and a significant reduction in goblet cell-associated mucin were stimulated following a single box exposure of histamine (10mM for 30mins) in the presence of mepyramine maleate (30mg/kg) to block H_1 receptor-mediated smooth muscle constricting effects (Fig. 6.3 and 6.6 respectively). Both lung function responses (Fig. 3.3) and histamine-induced mucin secretion (Fig. 3.6) were attenuated by pretreatment with the H_2 receptor antagonist ranitidine. It was therefore proposed that goblet cell mucin secretion could also been stimulated by H_2 receptor activation, resulting in airway mucus accumulation and a subsequent reduction in lung function.

A single box exposure of ATP in chronically OA challenged guinea pigs did not induce a significant reduction in sG_{aw} (Fig. 4.13) or a reduction in the mean % of AB/PAS-positive bronchiolar epithelial area (Fig. 4.14) compared to control. However, it was suggested that the lack of significance in both of these responses appeared to be due to large interindividual differences, possibly a result of the additional effects of ATP metabolites in the airways.

5'AMP did not induce any immediate change (up to 1hr) in lung function responses, compared to baseline in chronically OA challenged guinea pigs (Fig. 5.7). Sensitised guinea pigs however responded to 5'AMP with an early and late phase asthmatic response. The lack of response following the chronic OA challenge could be attributed to release of mast cell-stored mediators by previous OA challenges, leaving them exhausted of mediators for release by 5'AMP. 5'AMP did however, induce a significant reduction in goblet cell-associated epithelial stored mucin, although this was less than that observed following UTP exposure (Fig. 5.9). This result shows that adenosine (derived from 5'AMP) is a secretagogue, although the receptor subtype and whether it is A_3 receptor-mediated, as proposed by Young (2006) remains to be established. The result also shows that the degree of mucus secretion is insufficient to cause impaired lung function compared with UTP or histamine.

Inhaled UDP (1mM for 15mins) induced similar airway responses in chronically OA challenged guinea pigs as that observed following inhaled UTP (1mM for 15mins). UDP stimulated a significant reduction in the mean % of AB/PAS-positive bronchiolar epithelial area (Fig. 4.12) and a gradual reduction in lung function (Fig. 4.11) in chronically OA challenged guinea pigs. However, lung function responses were recovered more rapidly (at 45mins) following UDP exposure compared to nebulised exposure to its triphosphate counterpart, UTP (Fig. 4.11). These findings suggest that UDP may either be quickly degraded by airway ecto-enzymes or alternatively UDP may act as a weak/partial agonist to stimulate airway mucin secretion, which supports previous findings by Choi *et al* (2005) and Chen *et al* (2001).

The effect of nebulised exposure of combined secretagogues was investigated in Chapter 6. Nebulised UTP (1mM for 15mins) and histamine (10mM for 30mins) induced comparable lung function responses (Fig. 6.7) and reductions in the mean % of AB/PAS-positive bronchiolar epithelial area (Fig. 6.8), despite acting via two different receptors, the $P2Y_2$ receptor and H_2 receptor respectively. However, although a cumulative increase in goblet cell mucin secretion may be expected following a combined exposure of nebulised UTP and histamine, compared to a single secretagogue exposure, this was not observed in these studies (Fig. 6.8). This result suggests that there appeared to be a maximal release of mucin by these secretagogues, which left a possibly unreleasable portion retained in the goblet cells.

9.1.5 EXPERIMENTAL LIMITATIONS

Measurement of mucin secretion in animal models, as well as being tedious, can also tend to be inaccurate. Quantification of the area of AB/PAS-positive epithelial cells in groups of guinea pigs challenged with different exposure protocols allows assessment of goblet cell associated mucin secretion following secretagogue exposure. However, two groups of guinea pigs are required: one to measure epithelial stored mucin before secretagogue exposure and another to measure epithelial stored mucin following secretagogue exposure. The lack of internal control means that inter-individual differences in basal epithelial-stored mucin levels are not considered, resulting in potentially large s.e.m. values. Additionally, quantification of the AB/PAS-positive

epithelial area in histological sections of guinea pigs airways involves the measurement of a 2-dimensional area, rather than a volume of mucin. However, larger goblet cells may span the same area of bronchiolar epithelium in histological sections as smaller goblet cells, depending on their 3-dimensional shape. Therefore, the measurement of epithelial stored mucin in AB/PAS-stained paraffin sections of guinea pig lung may result in inaccurate findings or incorrect interpretation of results.

The quantification of BALF mucin concentrations also appears to have some experimental limitations. Although a significant increase in BALF mucin content was revealed in chronically OA challenged guinea pigs, compared to acutely OA challenged guinea pigs, secretagogue exposure in these animals induced no significant effect on BALF mucin content. This was inconsistent with the histological findings, which suggested goblet cell-associated mucin secretion following secretagogue exposure. It is unclear why this occurred. It was proposed that this might be due, once again, to a lack of internal control. Guinea pigs appear to have large inter-individual differences and therefore whilst the UEA-1 ELLA may be capable of measuring large differences in BALF mucin content (such as those observed following chronic OA challenge, compared to acute OA challenge), it cannot identify subtle changes. Alternatively, it may have been due to an incorrect choice of lectin. UEA-1 reacts with and identifies the fucose sugars of mucin oligosaccharides. It is therefore possible that mucin, secreted in response to secretagogues, has a small fucose content and will not be measured by the UEA-1 ELLA. Dot blot analysis of BALF lectin-positive content revealed an observable increase in HPA-positive material in BALF of secretagogue-treated animals compared to chronically OA challenged animals. HPA identifies a different mucin component, N-acetylgalactosamine, suggesting that HPA may be a suitable candidate to incorporate in a Sandwich ELLA for quantification of BALF mucin.

ATP, UTP, UDP and 5'AMP were all utilised as potential secretagogues. However, these nucleotides can be rapidly broken down in the body by membrane-bound ecto-enzymes, limiting their effectiveness in animal models. The enzyme-catalysed inactivation of nucleotides is coupled with the formation of breakdown nucleotides and nucleosides, which can also be pharmacologically active at alternative purinergic and pyriminergic receptors. It is also important to remember that the purity of commercially

available nucleotides may be questionable and contamination with other nucleotides or breakdown products is likely. The catabolism of nucleotides and possible presence of degradation products complicates the analysis of biological responses to nucleotides and it is important to remember that responses observed following nucleotide or nucleoside administration might also be due to their respective metabolites or phosphorylation products (Brunschweiger and Muller 2006).

In addition to the breakdown products of UTP, UDP and ATP, all of the chosen secretagogues have additional effects in the airways, other than mucin secretion, and this is arguably the main experimental limitation faced in these studies. These biological effects are detailed in the General Introduction and the introductions to individual chapters and include bronchoconstriction, bronchodilation, mast cell degranulation and effects on MCC and cough. These may all influence lung function responses and must be taken into consideration throughout these studies. Although the H_1 receptor (which is known to mediate histamine-induced bronchoconstriction) was inhibited throughout studies in Chapter 6, inhibition of additional receptors may have reduced the likelihood of secretagogue-mediated additional airway responses.

9.1.6 FURTHER WORK

With time and financial limitations, there were numerous further experiments that I would have liked to carry out. As discussed earlier, the development of the chronic OA challenge introduced the phenomenon of tolerance to these studies. However, there are multiple mechanisms of tolerance, mediated by a variety of inflammatory cells and mediators. The identification and measurement of such cells, mediators and antibody classes, as well as determination of the activation state of the inflammatory cells, in guinea pig BALF and blood samples would have provided more information regarding the mechanism of tolerance observed in the guinea pig of chronic asthma.

Additionally, as discussed in Chapter 4, despite demonstrating no UTP-induced bronchoconstriction in the normal guinea pig airway, UTP and additional secretagogues may induce bronchoconstriction following chronic OA challenge due to airway remodelling and potential altered receptor expression. Further investigation into the effect of secretagogue administration on airway smooth muscle in isolated tissue of

chronically OA challenged guinea pigs may provide additional evidence as to whether the secretagogue-mediated lung function responses were solely due to mucus secretion or also a result of bronchoconstriction of guinea pig airways. Furthermore, although P2 receptor-mediated mucin secretion was demonstrated in these studies, the specific receptor subtype remains to be established, possibly by $P2Y_2$ receptor selective agonists and antagonists.

The present studies reveal that goblet cell degranulation and mucin secretion occurred at a time point between 0mins and 1hr subsequent to secretagogue exposure. BALF mucin content was measured, using the UEA-I ELLA, at 1hr subsequent to secretagogue exposure. However, mucus can be removed form the airways either by mucociliary clearance, which involves the cilia-facilitated movement of mucus to the mouth to be expectorated or swallowed or by cough, which dislodges mucus from the airways. Therefore, it is possible that some mucus may have been removed from the airways at 1hr subsequent to secretagogue exposure. Further studies, to determine the exact time point of mucin secretion following secretagogue exposure could maximise BALF mucus content following secretagogue exposure.

Additionally, at 48hrs subsequent to UTP exposure, epithelial mucin accumulation is restored towards control levels (Fig. 4.10), suggesting that the epithelium's capacity to store increased amounts of mucin persists for at least 48hrs. However, due to limited research it is unclear whether this effect is short term or long lasting. Further studies investigating the time scale of mucin secretion and goblet cell-associated mucin production could provide valuable information regarding the development of a mucus hypersecretory phenotype.

9.1.7 CLINICAL RELEVANCE OF THESE STUDIES

Mucus hypersecretion is an important feature of several respiratory diseases including asthma. In the past, mucus was considered an innocent bystander of respiratory disease (Melton 2002). However, epidemiological studies suggest that mucus hypersecretion can have a dramatic effect on lung function, may lead to a loss of disease control and increased risk of mortality (Vestbo *et al* 2002). Recently, there has been intense interest in the impact that mucus hypersecretion and resulting airway obstruction has in

respiratory diseases such as asthma, COPD and CF. Studies by Agrawal *et al* (2007) have utilised a MARCKs-related peptide (Agrawal *et al* 2007) to investigate the effects of mucus secretion on lung function in a mouse model. The present studies, to my knowledge, confirm for the first time in a guinea pig model that excessive mucus secretion can have a detrimental effect upon lung function.

The mucolytic agent N-acetylcysteine and the expectorant guaifenesin are used for excess airway mucus, despite being widely criticised as ineffective. There are however several compounds which may promote mucus clearance from the airways or potentially inhibit the production of mucin and therefore may be used for the treatment of mucus hypersecretion. Potential compounds for mucus hypersecretion were summarised by Rogers (2004) and include neural inhibitors, inhibitors of MARCKs or Munc18 (which are involved in the exocytosis of goblet cell mucin granules), inhibitors of MUC gene upregulation or goblet cell hyperplasia (such as EGFR inhibitors) and anti-inflammatory compounds.

The OA model of chronic asthma, developed in the present studies, displays EAR, LAR, AHR and inflammation, as well as a mucus hypersecretory phenotype. The guinea pig model of chronic asthma developed in these studies may therefore provide a valuable model that may be used for the preclinical evaluation of potential pharmocotherapies for mucus hypersecretion, which are vital for the treatment of asthma and other inflammatory airway diseases.

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APPENDIX I

Poiseuille's Law: $\Delta P = \frac{8l\mu v}{\pi r^4}$ where ΔP= pressure drop due to friction

μ= viscosity

v= flow rate

l= length of tube

r= radius

Poisuelle's law describes laminar flow in smooth walled vessels where there is no appreciable turbulance. This equation can be used to describe pressure changes within the airways in response to changes in airway radius and airway length. Increased bronchoconstriction and airway secretions cause a reduction in airway radius. Additionally, as airflow approaches zero it becomes laminar.

Whole body plethysmography measured specific airway conductance (sG_{aw}) in unanaesthetised spontaneously breathing guinea pigs as described previously (Griffiths-Johnson *et al* 1998). Flow through the airway is driven by the pressure difference (P_d) between the upper airway pressure at the mouth (P_m) and the peripheral airway pressure at the alveoli (P_{alv})

$P_d = P_m - P_{alv}$

Airway resistance (R_{aw}) is defined as the relationship between instantaneous flow (v) and the pressure difference (P_d) between the mouth and alveoli.

$R_{aw} = P_d/v$

Airway conductance (G_{aw}) is described as the reciprocal of resistance

$G_{aw} = R_{aw} -1 = v/P_d$

To correct for interindividual differences in TGV between individual guinea pigs and therefore allow comparisons between different subjects, sG_{aw} is often used.

$$sG_{aw} = G_{aw}.TGV$$
$$sG_{aw} = v/P_d.TGV \quad \text{(i)}$$

During respiration, alterations in the pressure difference between mouth and alveoli allow air to enter and leave the lungs. To enable alterations in alveolar pressure and thus allow air to enter the lungs as the animal breathes the volume of the thoracic cavity increases. This results in a reduction in the air volume surrounding the animal and ultimately changes in pressure within the constant volume body plethysmograph. Boyles law states that in a sealed box, at constant temperature, changes in pressure are inversely related to changes in gas volume

$$P_1V_1 = P_2V_2$$
or
$$PV = (P + \delta P).(V-\delta V)$$

By multiplying out the brackets:

$$P.V = P.V - P.\delta V + V.\delta P - \delta P.\delta V$$

As $\delta P\delta V$ is negligible:

$$V.\delta P = P.\delta V$$

Changes in box pressure result form the difference in chest volume (V_c) and respired air volume changes (V_r) at atmospheric pressure (P_{atm}), corrected for saturated water vapour pressure (P_{svp}).

$$\delta Pd.TGV = \delta(V_c-V_r).(P_{atm}-P_{svp}) \quad \text{(ii)}$$

To describe laminar flow (by Poiseuille's Law) airflow must tend towards zero. This occurs during end inspiration and end expiration. In this study measurements were taken at end expiration.

By substituting equation (i) into equation (ii):

$$sG_{aw} = \frac{\delta v}{\delta(V_c - V_r).(P_{atm} - P_{svp})}$$

Utilisation of the Biopac data acquisition system allows measurement of both $\delta(V_c-V_r)$ and δv. $\delta(V_c-V_r)$ is the slope of the change in box pressure where flow tends towards zero (at end of expiration). It is then possible to record the simultaneous change in flow (δv).

Atmospheric pressure and saturated water pressure are known.

$P_{atm} = 1010$ cm H_2O

$P_{svp} = 63$ cm H_2O

Therefore sG_{aw} can be calculated from the following equation:

$$sG_{aw} = \frac{\delta[v]}{\delta [V_{box}].947.cf} \qquad s^{-1}cmH_2O$$

A correction factor (cf) must be used when calculating sG_{aw} to allow for the volume of the plethysmograph chamber displaced by each guinea pig.

$V_n = V_{box} - V_{gp}$ where V_n = net volume of plethysmograph chamber

$$cf = \frac{(V_{box} - V_{gp})}{V_{box}} \qquad \text{(iii)}$$

The volume of guinea pig (V_{gp}) can be calculated by the following equation

$V_{gp} = \frac{W_{gp}}{P.gp \text{ ltrs}}$ where W_{gp} = weight of guinea pig

$P.gp$ = density of guinea pig ($1.07\ kg.l^{-1}$) (iv)

By substituting equation (iv) into equation (iii):

$$cf = 1 - \frac{W_{gp}}{P.gp} . V_{box}$$

$$cf = 1 - \frac{W_{gp}}{5.885}$$

Therefore sG_{aw} can be calculated from the following equation:

$$sG_{aw} = \frac{\delta[v]}{\delta[V_{box}].947.(1 - W_{gp}/5.885)} \quad s^{-1}cmH_2O^{-1}$$

APPENDIX II

Publications arising from thesis

John, E.; Jackson, A.; Broadley, K.J. (2005). Mucus production and tolerance to inhlaed ovalbumin in a guinea pig model of chronic asthma. Proceedings of the British Pharmacology Society. PA2online. (4): p119.

John, E.; Jackson, A.; Broadley, K.J. (2006). Reductions in stored mucin and airway responses to secretagogues in a guinea pig model of chronic asthma. Proceedings of the British Pharmacology Society. PA2online. 4(2): p131.

John, E.; Jackson, A.; Sugar, R.; Growcott, E.; Broadley, K.J. (2007). Development of a guinea pig model of chronic asthma demonstrating a mucus secretory phenotype. American Journal of Respiratory and Critical Care Medicine. Abstracts Issue. 175: A464.